Genetic Engineering of Microorganisms for Chemicals

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Edited by

Alexander Hollaender

Associated Universities, Inc., Washington, DC

and

Ralph D. DeMoss, Samuel Kaplan, Jordan Konisky, Dwayne Savage, and Ralph S. Wolfe

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Proceedings of a symposium on Genetic Engineering of Microorganisms for Chemicals, held May 26-29, 1981, at the University of Illinois at Champaign-Urbana

This University of Illinois Symposium was generously supported by the Department of Energy, the Office of Naval Research, and the National Science Foundation, and the following industrial companies: Monsanto, Upjohn, A. E. Stanley, and Eli Lilly.

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This symposium was made possible through the most excellent cooperation and support of the Department of Energy, the Office of Naval Research, and the National Science Foundation. We are also grateful for the augmenting grants made from the Eli Lilly Company, Indianapolis, Indiana; the Monsanto Corporation, St. Louis, Missouri; the A.E. Staley Company, Decatur, Illinois; and the Upjohn Company, Kalamazoo, Michigan.

The authorities of the University of Illinois at Champaign-Urbana helped very much to increase the effectiveness of this symposium. The magnificent facilities of the campus were most pleasant and convenient. We acknowledge the planning efforts of our Scientific Advisory Committee, consisting of Irwin Gunsalus, Robert Rabson, John Reeve, Raymond Valentine, and Oskar Zaborsky. We appreciatively acknowledge the consultation of I.C. Gunsalus and Ralph DeMoss (Chairman of the Microbiology Department), as well as their Administrative Staff and the Office of Conferences and Institutes.

This symposium followed the conference on "Trends in the Biology of Fermentations for Fuels and Chemicals" which was held just six months earlier at the Brookhaven National Laboratory on Long Island, New York and which has also been published by the Plenum Publishing Corporation, New York. Together, these proceedings describe the present status of fermentation technology and bring out the newest aspects of microbial study which are of great promise in this most interesting field of genetic engineering.

I thank my Editorial Team of R. D. DeMoss, Samuel Kaplan, Jordan Konisky, Dwayne Savage, and Ralph S. Wolfe for their most effective editorial work and we appreciate the conscientious efforts of our Production Editor, Claire M. Wilson, and her associates who assembled the manuscripts.

Of course, the success of such a symposium is based especially upon the cooperation of its speakers and authors whose efforts we gratefully acknowledge in the composition of this volume.

Alexander Hollaender

FOREWORD

The normal course of most biologically catalyzed processes is tightly regulated at the genetic and physiological levels. The regulatory mechanisms are diverse, sometimes redundant, and it is becoming increasingly apparent that, at the genetic level, the range of mechanisms may be limited only by the permutations and combinations available. For each microbial cell, evolution appears to have resulted in maximized advantage to that cell, achieving regulatory balance.

Genetic engineering encompasses our attempts to perturb the genetic regulation of a cell so that we may obtain desired other than normal outcomes, such as increased product formation, or new product formation.

Following the groundwork established by a preceding symposium (Trends in the Biology of Fermentations for Fuels and Chemicals, Brookhaven National Laboratory, December 1980), the initial planning for this conference envisioned the juxtaposition of molecular genetic expertise and microbial biochemical expertise. The resultant interaction should encourage new and extended ideas for the improvement of strains and for the generation of new regulatory combinations to enhance microbial chemical production from cheap and abundant (including waste) substrates. The interaction should also demonstrate that new discoveries at the basic level remain essential to progress in genetic engineering. New genetic regulatory combinations require new studies of physiology and biochemistry to assure understanding and control of the system. New biochemical reactions necessitate new studies of genetic and regulatory interaction. Which systems are ready for exploitation? Which systems need further genetic refinement? Which systems should be extended to additional substrate utilization, and to additional or alternative product formation?

The proceedings of the Conference show that the raw ingredients are available for the broad application of genetic engineering of microorganisms. The manipulative genetic techniques, their problems and limitations, and the physiological controls, are clear in detail

for several types of microorganisms. The application desired is limited primarily by the repetitive work of selection and analysis. For many other organisms, the broad outlines of techniques are recognizable, and further detailed studies are certain to result in subsequent successful application. There remain many potentially useful systems, microorganisms and specific biochemical processes, in which only an inkling of appropriate methodology is available. If this conference has achieved its primary purposes, many of the participants will have recognized the usefulness of genetic engineering, and they will have already formulated plans for perturbing their chosen systems to generate and to improve the microbial formation of chemicals.

Plans for the Conference, development of the program, and requests for supporting funds were extensive, often seemingly endless. Those efforts were eased and smoothed by the most welcome participation of Alexander Hollaender, Ralph S. Wolfe and I. C. Gunsalus. Additional advice was always freely given by James A. Hoch, Jordan Konisky, and Sam Kaplan. The scientific success was assured in last-minute agreements by Douglas W. Ribbons, William B. Whitman and Paul S. Lovett, to replace late withdrawals from the program.

Technical success of the program was due almost completely to the outstanding efforts of Carol D. Holden, Mae A. Maxwell and Joline C. Henss of the Division of Conferences and Institutes of the University of Illinois, and of Claire M. Wilson of the Council for Research Planning in Biological Sciences.

Funding for this Conference was sought at a particularly inopportune moment in the life of the Federal government. Significant changes in national governmental leadership, together with attendant uncertainties in Federal agency budget planning resulted finally in a large gap in funding awarded. In view of the difficult circumstances, many thanks are due the Department of Energy, the National Science Foundation, and particularly to the Office of Naval Research all of which agencies responded affirmatively to requests for support. Funding also was provided by the Department of Microbiology, the School of Life Sciences, and the Campus Research Board, all of which are units of the University of Illinois. Special recognition and thanks are offered to Theodore L. Brown, Vice Chancellor for Research and Dean of the Graduate College, for his deep understanding and quick support of the Conference purposes and potential, and for his wise words of welcome opening the Conference.

The significant gap remaining in support funds, just one week prior to the Conference, was narrowed by contributions from midwestern industries in response to our last-hour plea for support. It is a considerable pleasure to acknowledge financial help from Monsanto, the Upjohn Company, A. E. Staley Company, and Eli Lilly Co.

Without their generous support, it would have been impossible to maintain the Conference at a meaningful and acceptable level of quality.

Finally the ultimate success of the Conference resides in the ability and cooperation of the speakers, the session chairmen, the panelists, the many participants and the editorial board, all of whom added important dimensions to the scientific quality, and to the future applicability of genetic engineering of microorganisms for chemicals.

Ralph D. DeMoss
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INTRODUCTION

Irwin C. Gunsalus

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The privilege of welcoming our colleagues, students, and friends to the Urbana-Champaign campus on behalf of the biochemical-chemical component is a pleasure matching in importance to the topic of this symposium, "Genetic Engineering of Microorganisms". The faculty of Biochemistry, Microbiology, and affiliated efforts are at your disposal, and I daresay our schools of Chemical and of Life Sciences who also hold a heavy stake in the production of useful chemicals from renewable resources, join me in this hospitality and welcome.

The roles of microorganisms in service to man, without his manipulation, have prospered to dwarf their more devious interactions. Many here will be aware that a narrow margin favored decisions to attempt to activate the process employing fossil fuel petrochemicals over ethanols as a primary feedstock for the American chemical industry. In retrospect, with the predictable and perhaps foreseeable exhaustion of the fossil fuel supply, the main and renewable resources of complex carbon residues must necessarily anticipate the products of photosynthesis.

You are in the center of one of the richest agricultural regions in the world. The soil, water, and climate are here, and no less important, the manpower -- at present, educated and vigilant. Much of this prime resource of the Midwest is derived from recent immigrant stock, in the sense of Will Rogers, who attributed the more serious problems of the country to the unusually liberal immigration policies of the American Indians. The numbers required to participate in the production of these renewable resources are still decreasing through the effort of the mechanical mule, a consumer of fuel only when producing useful work, willing to assume

the heaviest portions of the work load, but, unfortunately, not without the expenditure of fossil fuel. The sources of this energy-mobile fuel to plant, harvest, transport, and process the crops, the ancillary and not altogether minor expenditures to manufacture the machinery, fertilizer, crop protection chemicals, in aggregate, presents a magnitude in feedstock and energy requirements far beyond the concept of the large majority of university staff - scholar and student. The magnitude of energy currently expended as fossil fuel per acre of corn or soybean in Champaign County has been estimated in the order of $\sim$84 gallons of diesel fuel equivalent to approximately $100 per acre.

The emphasis of this conference is to increase the quantity and the quality of products through more effective, efficient, if you will, utilization of solar energy and renewable products thereof, and to enhance the processing by improvements in rate and total conversion. In the last thirty years, the infusion of genetics and molecular genetic science into the largely empirical considerations of microorganisms has brought forward new strategies, techniques, and concepts and has produced an impact of increasing proportion.

Our problem will, over the next three days, be introduced by the Plenary Lecture of Dr. Helinski to re-look at biology in industrial production. Of primary concern are the organisms which constitute the library of Nature; mindful of the capital expenditure required for plant construction; the importance of rate of turnover; the concentration of feedstock and product, and the costs of concentration to minimize energy and labor expenditure to realize, on the bottom line, pounds per day per dollar of capital outlay.

In complementation of Vice Chancellor Brown's greetings from the campus, we welcome the university and graduate college participants from the distant parts of America and abroad. We look forward to renew and renewed acquaintances, friendships, and information exchanges.

WELCOMING REMARKS

Alexander Hollaender

Associated Universities, Inc.
Council for Research Planning in Biological Sciences
1717 Massachusetts Avenue, N.W.
Washington, D.C. 20036

I am very pleased that we were able to plan this symposium on Genetic Engineering of Microorganisms for Chemicals less than six months from our previous symposium on Trends in the Biology of Fermentations for Fuels and Chemicals. The volume for the December proceedings is now available. At the time that we arranged for it, we thought that a second symposium, dealing with more specific chemicals should follow the more general program on fermentations and, as you can see from the program, it was possible to develop this theme.

There have been important new achievements in genetic engineering and the many new tools which have been developed can be applied to problems, not only for the laboratory investigator, but for industry. This present symposium shows the way, I think, that technology will evolve in this area.

Illinois, of course, is situated where many of the applications for fermentations have been very successful over the years and has the potential of becoming a leading center for new tools and their application. As a matter of fact, the middle western area was the center for such research about 30 to 40 years ago, until cheaper sources became available in different areas for chemical production from oil sources. It is clear that we will have to return to energy sources with which we started, including sunlight and the fermentation of agricultural products.

I want to impress upon all of the speakers that we really mean "business" when we say that we want to bring out the proceedings

in 100 to 150 days of the symposium as demonstrated by the now available proceedings of the Fermentations symposium which was held last December. This depends, of course, very much on the speakers not only submitting their manuscripts early, but also making their proofreading corrections promptly. With such cooperation, we hope to have this volume out before the end of the year.

PLASMIDS AS VECTORS FOR GENE CLONING:

PAST, PRESENT, AND FUTURE USE

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INTRODUCTION

The title of this conference in itself suggests the merging of several traditional disciplines of study (chemistry, biochemistry, microbiology, and genetics) with recent and dramatic developments in molecular genetics. The revolutionary breakthroughs of recombinant DNA research are providing unique opportunities for the genetic manipulation of microorganisms. One of the major objectives of this conference is to assemble scientists of diverse expertise to focus collectively on the problem of constructing microorganisms with new or greatly enhanced capacities to produce chemical substances of agricultural, industrial, and medical importance. For this purpose, it is necessary to consider the most effective means of merging our understanding of the physiological, biochemical, and ecological properties of microorganisms with the powerful technological developments that have occurred in molecular genetics. This paper will deal with plasmids, a key molecular ingredient for the genetic manipulation of microorganisms using recombinant DNA techniques.

Plasmids are extrachromosomal genetic elements present in a wide variety of naturally occurring gram-negative and gram-positive bacteria. These elements specify a large number of important bacterial traits. Early genetic studies on plasmid elements in gram-negative bacteria identified two major types on the basis of their overall genetic content; conjugative and non-conjugative. Conjugative or self-transmissable plasmids possess, in addition to information that is required for their autonomous replication, a functional set of genes that is required for bacterial mating

and the conjugal transfer of the plasmid or regions of the host chromosome. Non-conjugative plasmids are not self-transmissable since they lack this functional set of mating or transfer genes. However, many of these non-conjugative plasmids can be mobilized for transfer by a conjugative plasmid present in all the same cell.

Early studies on the physical nature of plasmid molecules were carried out on the conjugative plasmids (sex factor) F*lac* (1, 2), plasmid ColE1 which specifies the antibiotically active protein colicin E1 (3) and several antibiotic resistant (R) plasmids of *Escherichia coli* (4,5). The first demonstration that a plasmid element exists as a covalently closed circular (CCC) DNA molecule came from studies with the plasmid ColE1 (6). This circular duplex DNA molecule is approximately 6 kb in size and is maintained at 10-15 copies per chromosome equivalent in an *E. coli* cell. Occasionally multimeric (dimer, trimer) forms of this CCC DNA molecule are observed. The number of copies of plasmid ColE1 per cell can be greatly amplified by incubation of the cells carrying the plasmid in the presence of a high concentration of chloramphenicol (7-9). Subsequent studies with a variety of other plasmid elements present in both gram-positive and gram-negative bacteria have shown that the covalently closed circular DNA form is a common structural property of plasmid elements (Fig. 1).

A variety of relatively simple procedures have been developed for the isolation of plasmid molecules from bacterial cells (10). The development of these procedures was facilitated by early observations made on the CCC DNA form of the animal virus SV40 (11). These studies established certain basic physical chemical properties of the CCC DNA form of the DNA molecule including the differential binding of an intercalcating dye as ethidium bromide to CCC and open circular or linear duplex DNA. On the basis of this differential dye binding property the CCC DNA plasmid molecules can be readily separated from open circular or linear (chromosomal DNA fragments) DNA in a dye-CsCl density gradient (12). The dye-buoyant-density method is the most widely used procedure to obtain large amounts of purified plasmid DNA.

Plasmids not only can be readily isolated as intact DNA molecules from bacterial cells, but these purified molecules can also be re-introduced in *E. coli* and a variety of other bacteria and, thus, re-established in the plasmid state (13). This finding and the identification of restriction enzymes that cleave unique sequences of DNA (14, 15) were key discoveries in the development of plasmids as vehicles for gene cloning (Fig. 2) (16). Plasmids are not the only vehicles for replication or maintenance of foreign DNA in a host cell. Bacterial viruses have also been developed as gene cloning vehicles (17, 18).

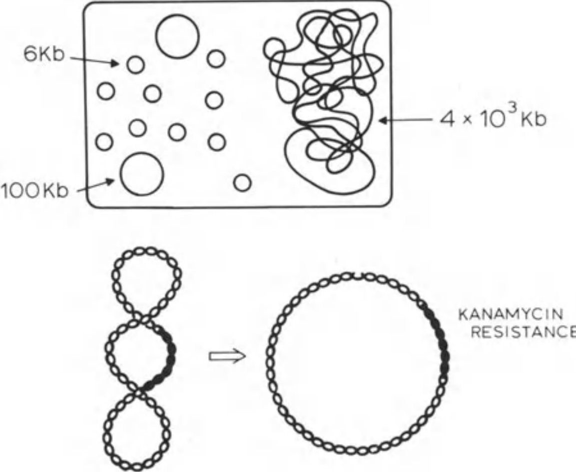

Figure 1. Circular DNA form of plasmids. Plasmids identified to date are present in the covalently-closed circular (CCC) DNA form. The CCC DNA form contains superhelical turns. If a single phosphodiester bond cleavage occurs in the molecule, the superhelical turns are removed and the molecule is converted to the open-circular (OC) DNA form. Size and copy number vary for different plasmid elements. Generally, the conjugative plasmids are larger than 75 Kb in size but exceptions are known. The *E. coli* chromosome is approximately 4 x 10^3 Kb.

CONSTRUCTION OF PLASMID CLONING VEHICLES

A wide variety of plasmid cloning vehicles have been derived from the naturally-occurring plasmids ColE1 and pMB1. Of these many derivatives, the plasmid pBR322 is the most commonly employed vehicle for gene cloning in *E. coli* (19). Like plasmid ColE1, pBR322 is a multi-copy plasmid whose copy number can be amplified by incubation of *E. coli* cells contaning the plasmid in the presence of chloramphenicol. By this procedure, large amounts of the plasmid including any gene insert that it contains can be obtained. With the construction of many derivatives of plasmids ColE1 and pMB1, an investigator has a large choice of selected markers and restriction endonuclease sites on a cloning vehicle for gene insertion. For example, the plasmid pMK2004, which was derived from pBR322, contains three selected markers (ampicillin, tetracycline and kanamycin resistances) and single restrictive sites for six different restriction endonucleases (Fig. 3) (10).

The small size of plasmid cloning vehicles derived from ColE1 and pMB1 is in part due to the finding that in the case of these plasmid replicons the extrachromosomal state is determined by a relatively small segment of the plasmid DNA [a sequence of

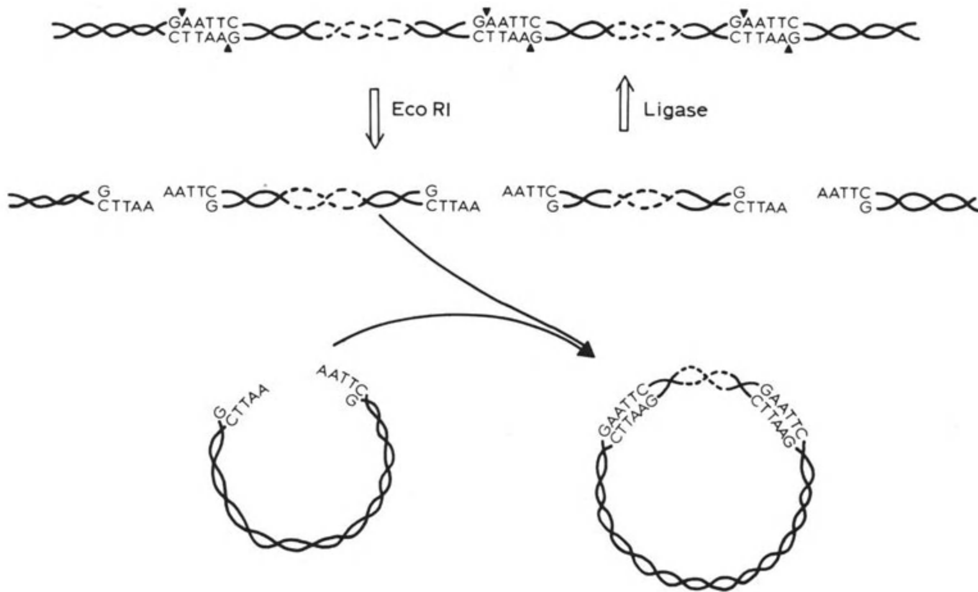

Figure 2. Steps involved in gene cloning. A restriction enzyme like EcoRI and a DNA joining enzyme (ligase) play key roles in this process.

approximately 580 base pairs (20)]. Both an origin of replication and nucleotide sequence information for the regulation of initiation of replication from this origin are contained within this 580 bp sequence. ColE1 and pBR322 do not encode for a protein that is required for replication (21-23). With cloning vehicles as pBR322 or pMK2004, which are multi-copy and amplifiable plasmids, a number of opportunities are provided for the isolation and manipulation of genes from a variety of sources (Table I).

While the effectiveness of ColE1-type plasmid cloning vehicles is limited to E. coli and closely related bacteria, the general concepts for the development of plasmid cloning vehicles for this organism have been extended to other host cells. For the development of cloning vehicles from most other plasmid replicons, however, considerably more information than an origin of replication must be provided in order to establish the extrachromosomal state. For example, unlike plasmids pBR322 or ColE1, a plasmid specified protein is required in addition to an origin of replication for the maintenance of the plasmid R6K in the extrachromosomal state (24,25). The antibiotic resistant plasmid R6K is 38 kb in size and is maintained as a multicopy

TABLE I
Use of Plasmid Vectors for Gene Cloning

PROVIDE LARGE AMOUNTS OF SPECIFIC GENES

GENES ARE EASILY RECOVERED FROM VECTOR

GENES CAN BE MANIPULATED _IN VITRO_

a. Base substitutions, deletion or insertion of nucleotides

b. Joining of genes to regulatory regions that promote gene expression of translation of mRNA in bacterial, fungal, animal, or plant cells

INTRODUCTION AND ESTABLISHMENT OF GENES IN A VARIETY OF HOST CELLS

a. _E. coli_ and other bacteria

b. Fungi (yeast)

c. Animal cells

d. Plant cells

plasmid (10-15 copies per chromosome equivalent) in _E. coli_ cells (Fig. 4) (26). This plasmid contains three unique origins of replication, designated α, β and λ (27-30). One can isolate the γ origin of replication as a discrete segment of DNA and replication can be initiated from this origin if the _E. coli_ cell is provided with an R6K structural gene that specifies the production of an initiation protein designated π (Fig. 5). A functional γ origin of replication consists of approximately 260 base pairs (31). It is of interest that the R6K γ origin contains seven tandem 22 bp direct repeats (Fig. 6) (32). Removal of three or more of the direct repeats results in a loss of origin activity (31). The complete γ replicon of R6K R6K, consisting of a discrete origin and the π structural gene, is 1583 bp in size (33). It is clear that a cloning vehicle derived from plasmid R6K is somewhat more complex than the ColE1 and pMB1 derivatives with regard to the amount of information required for establishing the plasmid state.

In the course of studies of the replication properties of a variety of plasmids of _E. coli_ and other bacteria, important basic information has been obtained which has been invaluable for the construction of plasmid cloning vehicles other than the derivatives of ColE1 and pMB1. Table II contains a selected list of such plasmid constructs. Whereas most of the

TABLE II
Selected Plasmid Cloning Vehicles

Plasmid	Replicon	Reference	Remarks
pBR322	pMB1	19	most widely used in E. coli
pACYC177	p15A	34	multi-copy plasmid compatible with ColE1 or pMB1 replicons
pDF41	F	10	low copy number plasmid cloning vehicle
pKN410	R1	35	temperature induced amplification of plasmid
pRK646	R6K	10	high biological containment since plasmid replication is dependent on R6K pir gene
R300B	R300B	36	broad host range
pKT231	RSF1010	37	broad host range
pRK290	RK2(RPI)	38	broad host range (binary vehicle system with pRK2013)

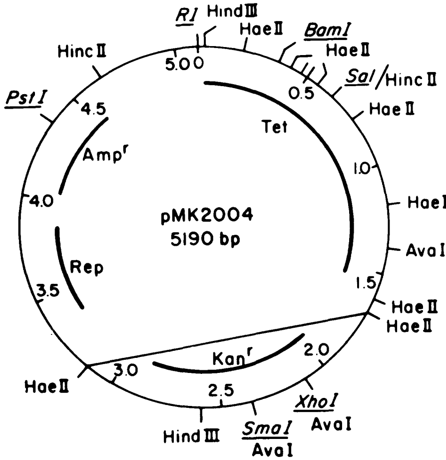

Figure 3. Restriction map of plasmid pMK2004. A segment of pBR322 that contains the plasmid replication region (Rep) and the ampicillin and tetracycline resistance genes was joined to a kanamycin resistant gene (10). A restriction site is underlined if it is present only once and if insertion into it does not inactivate plasmid replication.

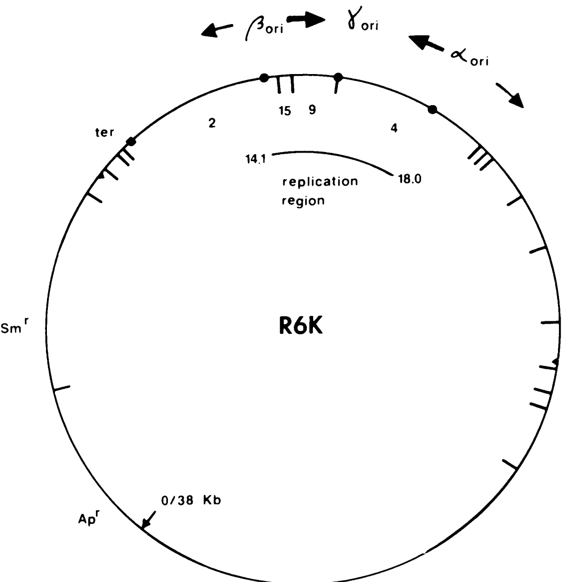

Figure 4. Physical and genetic map of R6K. The arrows indicate the sequential bi-directional mode of replication of origins α and β. 2, 15, 9 and 4 refer to specific Hind III fragments. The position of other Hind III sites, the Bam H1 (↑) site and EcoRI (▽) sites are also indicated; 'ter' refers to the terminus of replication.

plasmids that have been derived for gene cloning in the gram-negative bacteria are limited in their replication properties to E. coli and related organisms, a growing interest in gene cloning in other gram-negative bacteria of industrial, commercial, or medical interests has provided impetus for the development of broad host range plasmid cloning vehicles. Three broad host range plasmid cloning vehicles that are capable of maintenance in a wide variety of gram-negative bacteria are listed in Table II. Plasmid pKT231 is probably derived from the same replicon as R300B since plasmids B300B and RSF1010 (the parent of pKT231) are identical or very similar. Plasmid pRK290 (38) was derived from the incompatibility P-1 group plasmid RK2 which is similar but not identical to plasmids RP1 and RP4 of this group.

The incP-1 group of plasmids has been shown to be capable of self-transmission and stable maintenance in a variety of gram-negative bacteria, including the agriculturally important genera Rhizobium, Azotobacter, Agrobacterium and Pseudomonas. A binary vehicle, broad host range, cloning system was developed from plasmid RK1 (Fig. 7) by incorporating separately the transfer and

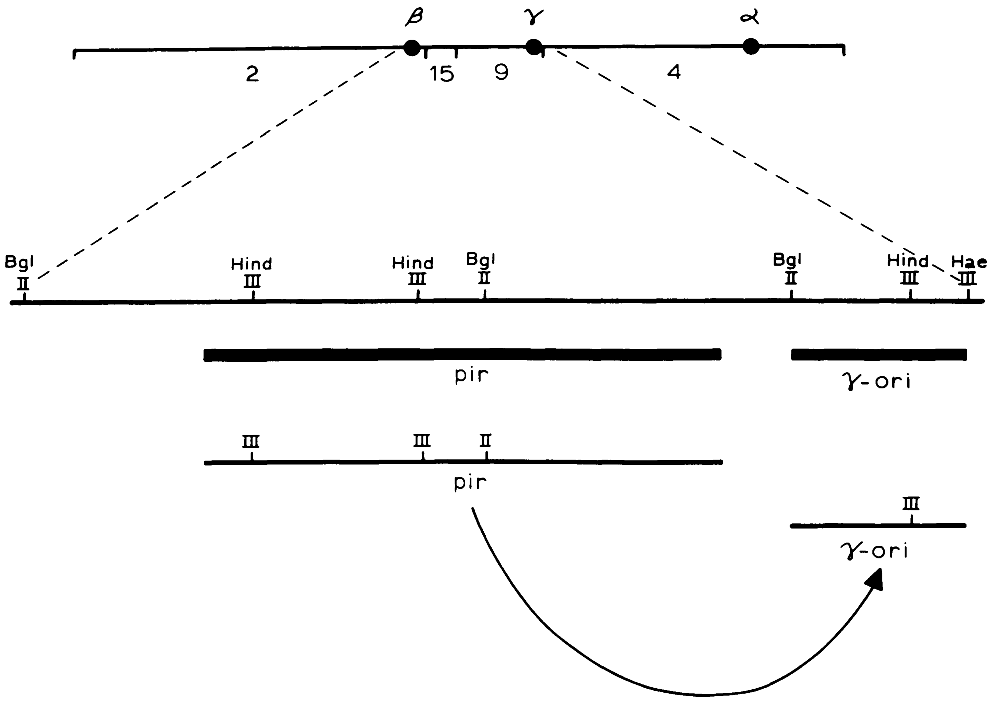

Figure 5. The π protein initiates replication at the γ-origin of plasmid R6K. Hind III fragments 15 and 9 and a portion of Hind III fragments 2 and 4 contain a structural gene (pir) which specifies a protein (π) that acts in trans to initiate replication from the γ origin.

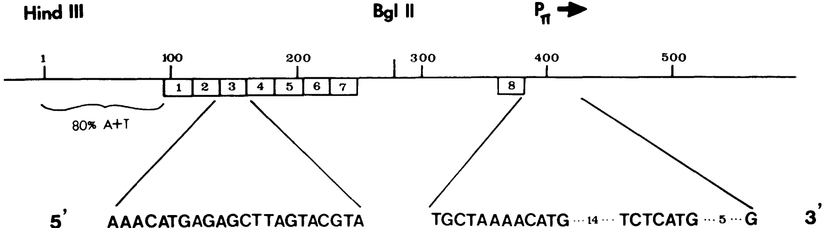

Figure 6. Major features of the nucleotide sequence of the γ origin region of R6K. Nucleotide sequences of one of the direct repeats and putative RNA polymerase recognition and binding sites in the promoter region (P_{π}) of the π gene also are indicated.

replication region of this plasmid into two different plasmid elements (38). Using recombinant DNA techniques, the three regions of the naturally occurring RK2 plasmid which are essential for replication, designated ori, Trf A and Trf B, have been joined in the construction of the plasmid cloning vehicle designated pRK290 (Figure 8). This cloning vehicle is a 20 kb tetracycline-resistant plasmid which is maintained at a copy number of 5-8 copies per chromosome equivalent in an E. coli cell. The plasmid is mobilizable but non-conjugative and has two unique restriction enzyme sites, Bgl II and EcoRI. Both of these sites are effective for the insertion of large DNA segments. pRK290 can be complemented in trans for conjugal transfer with the helper plasmid pRK2013 (Fig. 9). The helper plasmid is a kanamycin resistant hybrid containing the plasmid ColEl linked to a segment of RK2 DNA that specifies the transmissibility properties of this plasmid.

Although pRK2013 is self-transmissible, it cannot be maintained stably in bacteria distantly related to E. coli due to the narrow host range properties of the ColEl replicon. Foreign DNA inserted in pRK290, thus, can be transferred at very high frequency from E. coli to virtually all other gram-negative bacteria with this binary vehicle system (38). This property of the system is particularly valuable in view of the expected difficulty of transforming with purified DNA a wide variety of gram-negative bacteria other than E. coli. In addition, the introduction of foreign DNA on the plasmid pRK290 by means of conjugal transfer greatly reduces the restriction enzyme barrier that is present in many of the recipient gram-negative bacterial strains. The gene bank of the chromosomal DNA of the bacterium Rhizobium meliloti has been established in the plasmid vehicle pRK290 in E. coli and many members of this bank have been conjugatively transferred and established in a naturally occurring strain of R. meliloti (38).

The treatment of gram-negative and gram-positive bacteria as two well separated classes of prokaryotes draws additional support from the observation that no plasmid replicon has been found to be stably maintained in both types of bacteria. For this reason it has been necessary to derive plasmid cloning vehicles for gram-positive bacteria from naturally occurring plasmids of these organisms. A variety of plasmids from Staphylococcus aureus have the property of being stably maintained in Bacillus subtilus (39,40). Several of these naturally occurring plasmids exhibit the desirable cloning vehicle properties of relatively small size, single restriction sites and antibiotic resistance genes for selection. Certain of the plasmid derivatives constructed for gene cloning in Bacillus have been fused with E. coli plasmid vehicles like pBR322 in order to generate joint replicons that can be maintained in both gram-positive and gram-negative bacteria (Figure 10). Similar joint

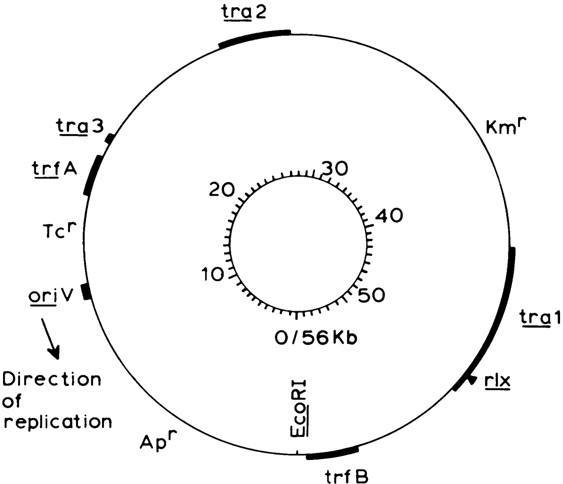

Figure 7. Physical and genetic map of RK2. In addition to the location of antibiotic resistance genes (Ap^r, Tc^r and Km^r), the sites of the origin of vegetative replication (oriV), trans-acting replication regions (trfA and trfB), conjugal transfer region (tra) and origin of conjugal transfer (rlx) are indicated.

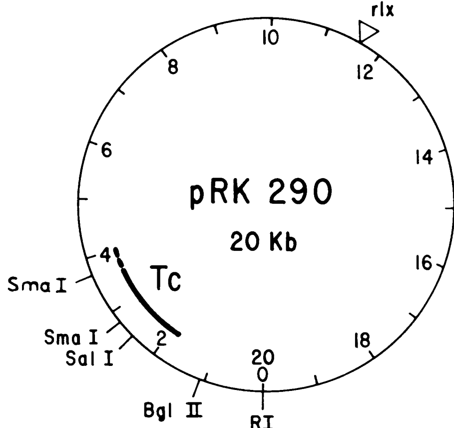

Figure 8. Restriction map of plasmid pRK290.

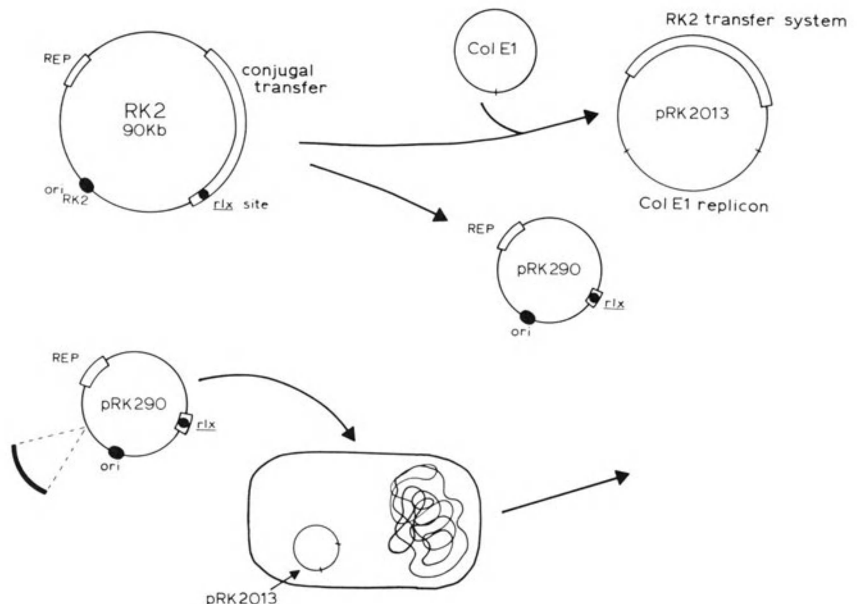

Figure 9. Broad host range, binary plasmid vehicle system. The properties of plasmids pRK290 (cloning vehicle) and pRK2013 (conjugative plasmid that promotes the transfer of pRK290 to a variety of gram-negative bacteria) are described in the text.

replicons have been constructed using E. coli plasmids and derivatives of naturally occurring plasmids found in gram-positive bacteria of the genus Streptomyces (41). With such joint replicon systems genes can be inserted into the cloning vehicle and the vehicle can serve as a shuttle for movement of the genes between a gram-positive organism and E. coli by transformation.

Similar shuttle plasmids have been constructed from the naturally-occurring small circular DNA or yeast cells (2 μ circles) and an E. coli plasmid replicon (Fig. 11) (42, 43). In addition to this circular DNA element that is naturally occurring in certain yeast strains and has been developed as a plasmid cloning vehicle, extra-chromosomal circular DNA elements capable of replication in yeast cells have been constructed utilizing segments of yeast chromosomal DNA which behave as a chromosomal replicator (44). While certain of these autonomously replicating fragments of yeast are maintained at high copy number, they are unstably maintained under non-selective conditions. The stability of these plasmids can be increased substantially by the insertion of centromere DNA sequences obtained from yeast chromosomes (45, 46). The presence of the centromere DNA also

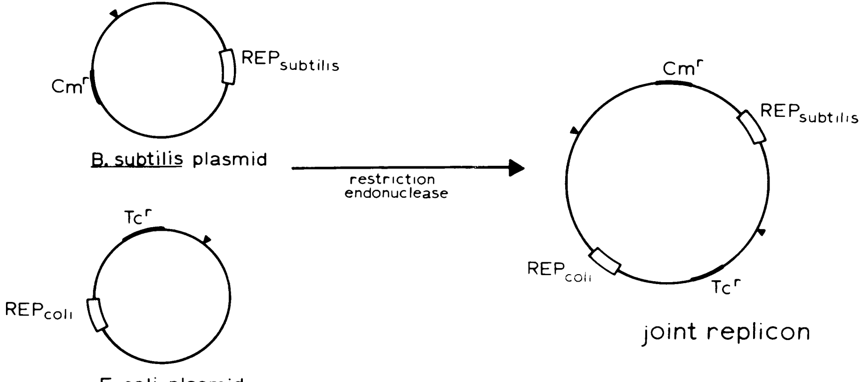

Figure 10. Shuttle plasmid for gene cloning in gram-positive and gram-negative bacteria. Joint-replicon, shuttle plasmids can be used for gene cloning between S. aureus, Bacillus species and E. coli.

reduces the copy number of the yeast plasmid element. These studies carried out with the yeast system are providing both valuable basic information on the replication and segregation of chromosomes in yeast and directions for future experimentation leading to the development of efficient plasmid-like cloning vehicles for a variety of eukaryotic cells.

ENGINEERING HIGHER YIELDS OF SPECIFIC GENE PRODUCTS

In the case of those multi-step biochemical pathways where a particular enzymatic component of the pathway is rate-limiting with regard to the production of a specific product, it should be possible to greatly elevate the intracellular level of the rate-limiting enzyme by either increasing the level of expression of the gene specifying that enzyme or increasing the cellular copy number of that gene. One direct method for increasing the copy number of a particular gene in a cell involves the insertion of that gene into a multicopy plasmid. For example, the insertion of the tryptophan operon of E. coli into the multicopy plasmid ColE1 resulted in greatly elevated amounts of the enzymes specified by the operon after derepression of the operon (47). A number of other instances subsequently have been reported where levels of specific enzymes in a cell have been greatly elevated by introducing a specific gene on a multicopy plasmid or phage vector by recombinant DNA techniques.

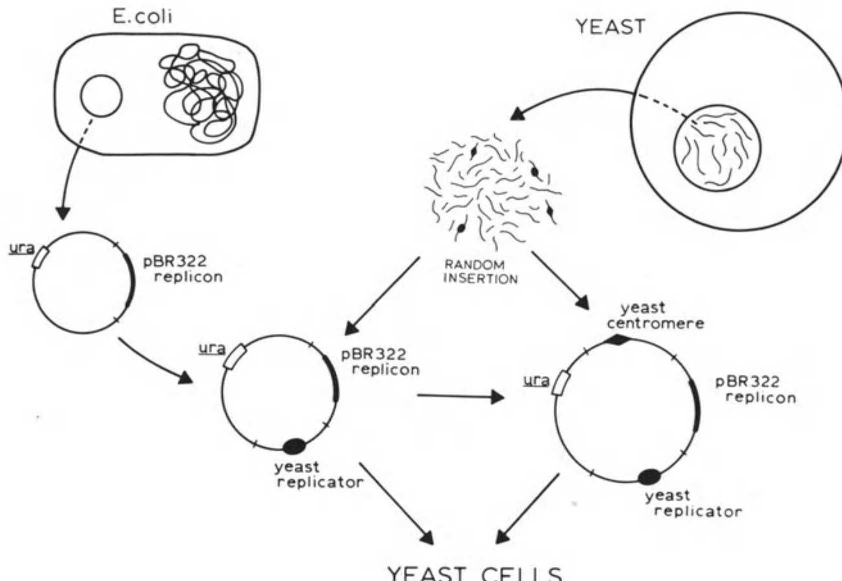

Figure 11. Plasmid cloning vehicles for yeast. Plasmids can be derived from segments of the yeast chromosomes that contain replication properties. The addition of portions of the centromere region of yeast chromosomes increases the stability of these plasmids in yeast cells.

Alternately, recombinant DNA techniques can greatly facilitate the introduction of mutations within a promotor (Fig. 12) (or repressor regulatory gene) that controls the expression of a specific gene or be used to fuse a specific gene with a naturally occurring and highly active promotor in order to amplify the amount of that gene's product. The latter case of fusion of a specific gene with a known promotor frequently has been used to obtain expression of an isolated gene in a foreign cytoplasmic environment. For promoting the expression of a eukaryotic or prokaryotic gene in an E. coli cell by fusion with an E. coli promotor, four well defined E. coli promotors have been used (Table III, see Ref. 48). These include the promotors of the lac operon of the E. coli and the ampicillin resistance gene of pBR322 which codes for the enzyme β-lactamase. These promoters have been highly effective in specifying high levels of production of protein specified by a variety of prokaryotic and eukaryotic genes. In addition to these constructions which are directed at overcoming transcriptional barriers, a number of recombinant DNA experiments have been carried out to bring the translation of transcripts

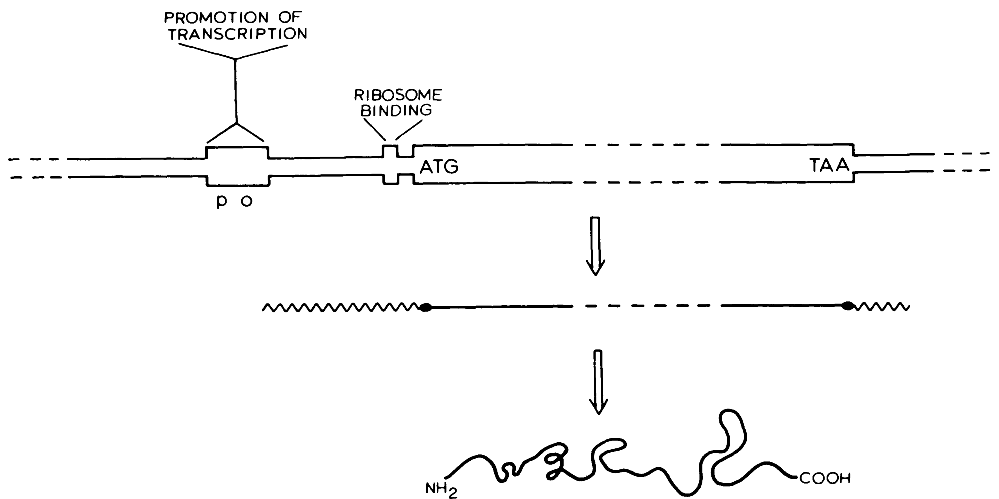

Figure 12. Signals for transcription and translation of prokaryotic genes. The relative positions of the promoter and ribosome binding sequences of nucleotides and the translational start and stop signals are indicated.

TABLE III
Plasmid Cloning Vehicles Promoting Expression

Promoter	Plasmid	Replicon
$\underline{P}_{\underline{lac}}$	pB6F120	pRSF2124
$\underline{P}_{\underline{L}}$	pHUB4	pMB1
$\underline{P}_{\underline{trp}}$	pAS621	pMB1
$\underline{P}_{\beta\text{-}\underline{lact}}$	pBK322	pMB1

of gene inserts under the control of translation initiation signals on the plasmid. This becomes particularly important for the expression of the foreign gene in a cytoplasmic environment where both specific transcriptional and translational signals must be efficiently recognized for maximum expression of the gene (Fig. 12).

An excellent example of bringing the expression of a complete eukaryotic gene under the control of both a promotor and a ribosome binding site of E. coli is the reported case of expression of the human growth hormone in E. coli (49). In these experiments, a Hae III fragment coding for amino acids 24-191 of the human growth hormone, including its C-terminus, was fused to a chemically synthesized DNA fragment encoding for the N-terminal amino acids of this protein. The fused DNA segment was inserted into a plasmid carrying the lac promotor and the β-galactosidase ribosome binding sequence. The hybrid plasmid containing the proper spatial arrangement between regulatory elements and the AUG starting codon specified the production of 186,000 monomers of the growth hormone per E. coli cell. Similar high levels of expression of a gene in the form of a protein product have been obtained with other well defined E. coli promotors. In the case of the β-lactamase promotor, advantage has been taken of the fact that the product of this gene is a periplasmic protein whose transport properties are determined by its N-terminal sequence. Fusion of the cDNA sequence encoding for rat pre-proinsulin to the N-terminal end of the β-lactamase gene resulted in a proinsulin fusion protein that was transported through the periplasmic space (50).

The concept of creating a fusion product between a known promotor and ribosome binding site of E. coli, the N-terminal codons of a structural gene and a DNA segment containing a foreign gene, has led to the construction of plasmid cloning vehicles of potentially general applicability. For example, the plasmid cloning vehicles pPCØ1, pPCØ2 and pPCØ3 carry on a pBR322 replicon a single EcoR1 site allowing the fusion of DNA sequences on a EcoR1 fragment with N-terminal sequences of the β-galactosidase gene in all three translational reading frames (51). Similarly, as shown in Figure 13, a single plasmid cloning vehicle has been constructed that permits the insertion of a foreign gene into any one of three possible reading frames depending upon which restriction site in the cloning vehicle is used downstream from an E. coli ribosome binding site and a highly active promotor (tryptophan promotor) (52).

It is clear that most of the effort to date has been directed at promoting the expression of cloned genes in the bacterium E. coli. As part of the success of these studies in E. coli, a number of basic concepts have been derived for the similar development of plasmid cloning vehicles that promote the expression

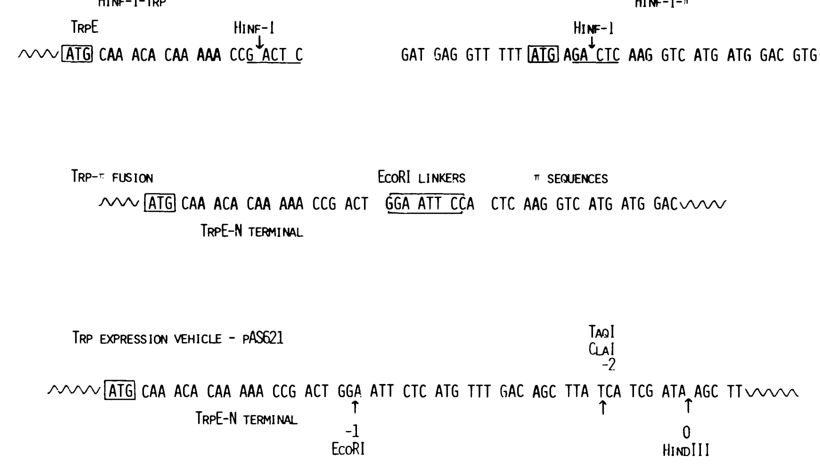

Figure 13. Plasmid cloning vehicle facilitating insertion of genes in all three translational reading frames. Plasmid pAS621 contains the efficient tryptophan operon promotor and the N-terminus of the TrpE gene. Using one of the three restriction enzyme sites indicated DNA segments can be inserted in all three possible reading frames for translation (52).

of gene inserts in other gram-negative bacteria, gram-positive bacteria and the cells of fungi, animals and plants. With the availability of plasmid cloning vehicles for different bacterial, fungal and animal cells, it becomes increasingly important to obtain basic information with regard to the structure of regulatory regions and ribosome binding sites that are functional in cells other than *E. coli* in order to design vehicles that promote the expression of genes in a variety of host cells.

FUTURE DIRECTIONS FOR THE CONSTRUCTION OF PLASMID CLONING VEHICLES

During the past five years there has been the development of a variety of plasmid cloning vehicles in bacterial cells. Substantial progress has also been made in the development of similar gene cloning vehicles in eukaryotic cells. We can fully expect an intensification of efforts in this direction leading to the construction of a wide variety of plasmid elements designed for establishing genes in the extrachomosomal state in virtually all organisms of agricutural, industrial or medical importance including bacterial, fungal, animal and plant cells. In addition to providing the means for establishing genetic information in a

variety of cell types, certain of these plasmids will be designed for carrying out specific objectives as, for example, a bacterial plasmid cloning vehicle that fuses specific information on the N-terminal end of a protein product for the purpose of transfer of that protein product across cellular membranes. There also will be increasing emphasis on the development of plasmid systems where the expression of a particular gene insert will be conditional, i.e., controlled in response to a change in the internal or external environment of the cell. Plasmids may be developed, for example, for the expression of a gene only under conditions of environmental stress and where the product of the induced gene protects the cell or organism against drought, high salinity, or extremes in temperature. It will also become increasingly important to develop plasmid vehicles that promote the integration of a cloned gene into a specifid site or domain within the chromosome of a fungal, plant or animal cell. The site-specific integration of a gene cloned by recombinant DNA techniques may be critical in providing for the expression of that gene at a particular stage in the development of a eukaryotic organism in order to maximize the beneficial effects of the gene product. As we obtain a greater understanding of the basic mechanisms for the control of gene expression in prokaryotic and eukaryotic organisms, the possibilities for the engineering of specific genes and cloning vehicles will greatly expand. The methodologies for carrying out these genetic manipulations are being improved substantially. This has in turn increased the level of sophistication and kinds of genetic manipulations that can be carried out. These developments have not only opened many possibilities in the applied area or biology, but also have provided for innovative new approaches towards an understanding of basic biological processes.

CONCLUDING COMMENTS

The development of recombinant DNA technology in university laboratories has provided powerful new tools for the biological sciences. These advances come at a time when societal problems (food, energy, health care and environmental stress) are increasing for the majority of the world population. Opportunities for imaginative and creative solutions to some of these problems have never been greater. However, while recombinant DNA technology provides revolutionary new approaches to problems, the success of the application of these techniques depends on the continued expansion of the broad informational base of the biological sciences and an adequate level of resources including sufficient numbers of trained individuals. This is an unprecedented period of opportunities for the biological sciences. It is unfortunate that it is also a period of increasingly severe limitations on the federal funding of research and the training of young men and

women in graduate programs in the biological sciences. The private sector of our economy must take on a substantial role in supporting basic and applied research and graduate programs in the biological sciences in the universities. It is only through universities, private industry and the federal government working together to promote basic and applied research and a free exchange of ideas that we will succeed in applying these powerful new techniques to our pressing national and global problems. This conference is one important mechanism by which the various sectors of the scientific community can interact. There are a number of other levels where this interaction also must occur if we are to take advantage of these revolutionary developments in the biological sciences for the benefit of society.

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STRATEGIES: TECHNIQUES AND MOLECULAR SYSTEMS

CHAIRMAN'S INTRODUCTION

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In beginning this meeting of the Ways and Means Committee which will last throughout the day, if I have any contribution from a good many years of involvement in gene transfer, it is to comment on the ideas that we used to hold in the past which have had to be collapsed or modified as we extended them as bridges into the new domain of genetic engineering. We used to consider DNA as metabolically stable, an unmodified substance, something like an aged grandfather living in the family, being served by all the family, influential, but remote from life in the kitchen. We now know how many ways DNA is participating, being subject to exchange, excision or modification, etc., as it serves its functions. We thought that very few kinds of cells could take up DNA. We used to say there are just two or three transformable species; *E. coli* for example was "not transformable," unless you accepted reports by Boivin and Vendrely about 1948 on very special strains, whose transformations could not be repeated by other people. We thought that the genes could only be transferred into pre-existing sites to which they were already homologous. Our approaches then and now considerably depend upon concepts about what is called the unity of biochemistry. Stimulated by Helinski's insightful and exciting talk, I have tried to reconsider just what we then meant and what we would now mean by the unity of biochemistry. We used to mean that the pathways of biosynthesis for metabolites in a wide variety of creatures were very much the same. I venture to say that we are not satisfied with this generalization today. The very purpose of introducing genes into another organism partakes of the idea that at least some elements in its pathways are not there and it would be advantageous or useful to explore them in a new environment. Perhaps inserted as part of a pathway that does not otherwise exist, they may remain isolated and, therefore, all the more isolatable. Some of the very difficulties we experienced in our old

transformation days, also affecting the new gene transfers, come from the refractivity of cells to DNA uptake. When you are trying to introduce a DNA or plasmid into a new cell, the first obstacle this element meets is the outer envelope, a highly differentiated set of products of particular pathways, likely to come out unique for each cell type.

In fact, we have come to realize that for preserving the integrity of the various species, nature has relied very much on compartmentation and boundary layers, made of just such biosynthetic products. Now that we have learned to bypass such barriers, we find that heterologous kinds of DNA have often a significant potential for instituting genetic change by stimulating "repair," or being inserted or copied. This is because the place where biochemical unity is most profound and impressive is in the near universal genetic code, and the ribosomes and other apparatus for translating its messages. Thus, it is not the enzymes making either the small metabolites - in the metabolic pathways - nor those making the macromolecules, at least it is not their structures, which are so unified. But the latter group, making DNA's, proteins, etc., have had to develop a "class-action" type of specificity, treating any of a group of nucleotides or amino acids as equivalents. And it is these class-action processing systems that are most susceptible to outside manipulation. Our genetic engineering consists to a large degree of running some of the not-so-uniform coded messages into the nearly uniform apparatus and letting them become entangled.

Another site of near uniformity is to be found in the lipid bilayers of cellular unit membranes. Once they are in protoplast form they are stripped of most of the species- and cell-specific barriers. Though still invested with some scant population of membrane and trans-membrane proteins, they are sufficiently alike to be able to be fused promiscuously with each other. Later in this session, Dr. Alfoldi will review his own, and some of our own work showing that bacterial protoplasts can be so fused, with interesting genetic consequences. Another uniformity resulting from protoplasting is the opening up of a variety of organisms, including yeasts, to direct transformation by DNA of polyethyleneglycol-treated spheroplasts. This, too, will be reflected in our symposium reports.

Alikeness or difference: these are relative matters, and depend upon the point of view. But, I repeat, our experimental approaches and our very opportunities are determined by our points of view.

Some of these changing views were beginning to come up in 1965. I speak of this date, because in what is for me quite personally an interesting coincidence, the last time I visited this campus, 16 years ago, I delivered the Wilhelmina Key Lecture before the American Society of Heredity, under the title, Portents for a Genetic

Engineering. I spoke mildly against treating human cells with DNA as premature (surely it then was), but pointed out some of its imagined attractions. There was quite a response from the press around the country, and many letters from the public, ranging from wistful admiration to cynicism, to outrage. I spoke mostly of "genetic intervention" because I pictured it as a private pact between donors and recipients and not at all like engineering. But I did suggest that viruses or similar vectors would be sought to raise the efficiency.

We were far from visualizing our present possibilities back then; much has since changed. Now we can all look forward to this Symposium and the prospects it will reveal. The process has clearly become a cleverly engineered undertaking rather than a bit-by-bit manipulation. Its day has come - and now the floor is yours.

CLONING VECTORS DERIVED FROM BACTERIAL PLASMIDS

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PLASMIDS AS FUNCTIONAL MODULES

Bacterial plasmids are a diverse group of extrachromosomal genetic elements which are found in a wide variety of procaryotes. An examination of a large number of naturally occuring plasmids has led to the conclusion that they have evolved through a process which can be referred to as "modular evolution". In this process, various functional units (such as those involved in conjugal transfer, antibiotic resistance and colicin production) have been combined with a replicating segment (replicon) to produce a composite plasmid. Further analysis has revealed that these functional units or modules are often transposable i.e., they can move from site to site in the absence of homologous recombination (40). Due to transposition, this process is dynamic, in that modules can be added or deleted to allow for the rapid evolution of more fit plasmids.

A typical example of this modular structure can be seen by comparing several members of the FII incompatibility group (Fig. 1). The plasmid R538-1 is composed of at least three distinct modules (1). One of these is involved in transfer and contains all the genes required for the conjugal transfer of the plasmid from one cell to another. Another module contains the genes for antibiotic resistance closely linked to each other and flanked by direct repeats of the insertion element IS1. The third module contains the genes required for autonomous replication of the plasmid.

An examination of the structure of plasmids R1 (11), R100 (46) and R6 (61) reveals that their organization is similar to that of R538-1. However, they do contain additional transposable

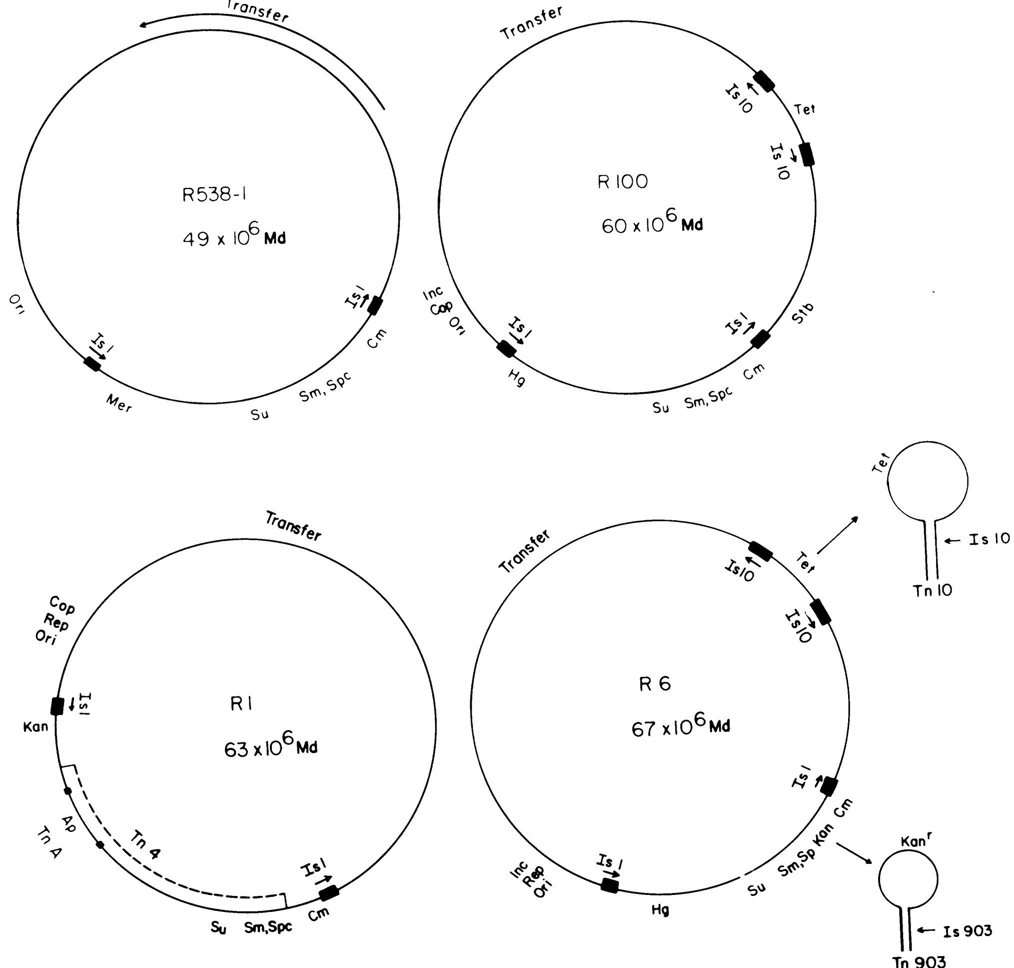

Figure 1. A comparison of the structures of plasmids R538-1, R100, R1 and R6. The insets by R6 demonstrate the typical stem and loop structures which single-stranded DNA from a region bounded by complementary 1S sequences can form by self hybridization.

antibiotic resistance markers. These include tetracycline resistance, Tc^R (Tn10), present in R100; kanamycin ardampicillin resistance, Km^R and Ap^R (Tn3), present in R1; and Tc^R (Tn10) and Km^R (Tn903) present in R6. The kanamycin resistance is present in different locations in R6 and R1.

This modular arrangement which is found with naturally occurring plasmids has greatly simplified the development of plasmid cloning

vectors. Since functional regions are clustered, they can be readily disassembled and assembled in the laboratory with restriction enzymes in much the same way that they have evolved by utilizing IS sequences and other transposable genetic elements in nature.

In the following sections, the components of plasmid cloning vectors involved in replication segregation, and selection will be briefly reviewed. The final section will summarize the properties of a number of plasmids commonly used for general DNA cloning, regulated expression of proteins from cloned DNA and detection of transcriptional signals on cloned DNA.

Replication Modules

The essential region for the construction of a plasmid vector is involved in replication. Although all of the factors involved in control of replication are not understood for any plasmid, this has not seriously hampered the development of plasmid cloning vectors. However, as our knowledge of the control of plasmid replication increases we can expect to have improved vectors in which the copy number (i.e. the number of plasmid molecules per cell) can be precisely regulated.

ColE1 Type Plasmids. The most commonly used plasmid vectors in *E. coli* are derived from ColE1 and ColE1-related plasmids pMB1 and p15A. The minimal essential replicon (MEP) of pMB1 comprises about 580 nucleotides (5). This region contains the site where initiation of replication occurs and codes for an RNA molecule of 104 nucleotides (42,36). This RNA apparently has a role in control of copy number since mutations in the RNA coding region lead to greatly increased copy number (50). No plasmid encoded proteins appear to be involved in ColE1 replication (26,38). Although ColE1 is normally present in about 25 copies per cell (20), the addition of chloramphenicol to logarithmically growing cells leads to an increase in the ColE1 copy number to as high as 1000-3000 copies per cell (19,20). This amplification greatly facilitates the isolation of large amounts of plasmid DNA. The pBR series of cloning vectors developed by Bolivar and co-workers is derived from pMB1, a close relative of ColE1.

IncFII Plasmids. The region of the IncFII plasmids R1, R6 and R100 required for autonomous replication are localized on a segment 3 kb in size (3,39,46,48). This region contains the origin of vegetative replication and the genes involved in incompatibility and copy number control. A number of cloning vectors have been derived from the IncFII plasmid R1drd19. R1drd19 is normally present in low copy number (3-5 per cell). However, a number of mutants have been isolated in which replication is temperature dependent (63). In strains carrying these mutant plasmids, raising the temperature results in uncontrolled plasmid replication which eventually leads to cell death. These so-called runaway replication mutants have a

number of features which make them useful as cloning vectors. In particular, since they can be amplified by temperature, rather than by inhibitors of protein synthesis, large amounts of protein can be produced from genes carried by these plasmids. Several derivatives of these runaway replication mutants have been constructed, and will be discussed below.

IncFI Plasmids. Cloning vectors have also been derived from the F-factor, a member of the FI incompatibility group (37). The F-factor is normally present in only 1-2 copies per cell. Small self-replicating plasmids have been derived from F and combined with several different antibiotic resistance genes. Because of their low copy number, these F derived vectors are useful for cloning of genes which might be lethal if present on multicopy plasmids.

IncPl and Q Plasmids. In general, the plasmids described above will replicate only in the enterics. Plasmids of the Pl and Q incompatibility group have a much broader host range. The Pl plasmids can conjugally transfer into, and replicate in, a large number of gram negative (gm^-) bacterial species. These plasmids, therefore, have the potential to be used as cloning vectors in a large number of gm^- organisms. Due to their efficient conjugal transfer system, this usefulness can be further extended to gm^- organisms which are not normally transformable. In these cases the initial cloning can be carried out in *E. coli*, followed by conjugation to introduce the gene into the organism under study. A cloning vector system suitable for such use was recently described by Ditta *et al*. (25).

In contrast to the ColEl, FI and FII Inc plasmids described above, the essential replication genes of the PI Inc group plasmids are not clustered, but are found in three different locations on the plasmid genome (29).

Stable Segregation Modules

In some cases, small self-replicating plasmids isolated from larger plasmids are less stable than the parent replicon. Meacock and Cohen (44) identified a region referred to as the *par* locus on plasmid pSC101 which was required for stable partitioning of the plasmid at cell division. A similar region referred to by Rownd as the stability locus (*stb*) has been identified on plasmid R100 (46) and on plasmid Rl (*par*) (47). Since *par* is not plasmid specific, it could presumably be used to stabilize different plasmid replicons (44).

Drug Resistance Modules

To obtain an appropriate plasmid cloning vector, the replication region must be combined with genes which can be readily

TABLE I

Plasmid Determined Antibiotic Resistances

Drug	Mode of Drug Action	Mechanism of Resistance	Reference
Chloramphenicol	inhibition of protein synthesis	drug detoxification by chloramphenicol transacetylase	54
Erythromycin-Lincomycin	inhibition of protein synthesis	methylation of ribosomal RNA to prevent drug binding and action	41
Hg(II) and Organomercurials	inactivation of protein through interaction with sulfhydryl groups	reduction of Hg(II) to Hg(0) (methallic mercury) which is rapidly removed by volatilization	57
Neomycin, Kanamycin, and similar Aminoglycosides	inhibition of protein synthesis	durg modification resulting in reduced drug uptake	24
Penicillins and Cephalosporins	inhibition of cell wall synthesis	drug detoxification by β-lactamase	52
Spectinomycin	inhibition of protein synthesis	drug modification resulting in reduced drug uptake	27
Streptomycin	inhibition of protein synthesis	drug modification resulting in reduced drug uptake	58
Sulfonamides	competitive inhibition of dihydorpteroate synthase	production of sulfonamide-resistant dihydropteroate synthase	65
Tetracycline	inhibition of protein synthesis	interference with drug transport into the cell	30

selected for. Antibiotic resistance genes have proven to be the most useful for this purpose for several reasons. These include: 1) since one is dealing with the conversion of a sensitive to a resistant phenotype they afford a positive selection; 2) most bacterial strains are sensitive to at least some antibiotics; 3) the genes involved in antibiotic resistance are well characterized and, in many cases, unique restriction fragments carrying the entire gene and promoter region have been identified thereby simplifying the introduction of a functional gene into a cloning vector; 4) resistance is expressed over a wide host range, since, in many cases, the mode of action involves modification of the antibiotic rather than alteration of the sensitive target. Several exceptions to this generality exist.

A list of some of the commonly used antibiotic resistance genes and their mode of resistance is given in Table I.

PLASMIDS AS CLONING VECTORS

General Usage Vectors

Table II lists a number of the plasmid cloning vectors which have been used in *E. coli*. Most have been derived from either ColE1 or the ColE1 related plasmids pMB1 and p15A. In general, a replicon has been combined with one of the antibiotic resistance genes described in Table I.

Some plasmids whose replication can be induced by heat are described in Table II. Most have been derived from R1drd19. An exception is pKC16 which contains the lambda N, O and P genes cloned in plasmid pBR322 (51).

Vectors Which Allow Regulated Gene Expression

Another aspect of cloning vector design and construction involves the regulated synthesis of protein from cloned genes. This really involves two aspects: 1) a repression system to avoid the production or overproduction of gene products which might be detrimental to the cell and 2) an induction or derepression mechanism to allow high level production of the cloned gene's product, when this is desired.

Although antibiotic resistance genes are extremely useful as selectable markers, they are not highly regulated. Several of the antibiotic resistance genes, including chloramphenicol acetyl transferase, streptomycin adenyl-transferase and kanamycin-neomycin phosphotransferase are under cyclic AMP control. However, they still show a significant level of constituitive synthesis which is only regulated over about a 2-fold level by c-AMP (33,62). Of the other commonly used antibiotic resistance genes, the Tc^R gene

carried by Tn10 is inducible by low levels of tetracycline (60), while the synthesis of β-lactamase (involved in ampicillin resistance), appears to be constituitive (52). The relatively small variations in the level of protein expression which these genes exhibit make them inappropriate sources of regulatory elements for precisely controlled differential expression of cloned product.

The most thoroughly studied genes, which are highly regulated, are those involved in lactose utilization (lac operon), tryptophan biosynthesis (trp operon) and bacteriophage lambda development. Sufficient genetic and biochemical data has been obtained to allow the ready adaptation of these genes as regulators of expression of other cloned genes. A list of some of the vectors which contain these regulatory elements is presented in Table III.

Vectors with Lac Operon Regulation. The lac operon consists the repressor gene (lac i), the operator-promoter region and the structural genes for β-galactosidase (lac z), lac permease (lac y) and transacetylase (lac a) (8). The synthesis of β-galactosidase is negatively regulated by the lac repressor coded by the lac i gene and positively regulated by c-AMP and c-AMP receptor protein. The relevant segments for the application to expression plasmids are lac i, the operator-promoter region and the amino terminal region of the lac z gene. Literally hundreds of mutations which effect the level of β-galactosidase have been isolated and mapped in this region (8).

The lac operator-promoter region has been used in several ways to obtain expression of heterologous genes. In one of these, a 95 bp restriction fragment which carries the lac promoter and the lac z ribosome binding site (SD sequence) was inserted at various distances from the ATG start codon of the gene to be expressed (7). Analysis of these fusions suggested that the optimal distance between the SD sequence and the f-met codon was 7-14 nucleotides. A generalized method to optimize expression by varying the distance between the SD sequence and the protein start codon has been described by Roberts and Ptashne (53).

A second type of construction utilizing this region involves the 203 bp HaeIII restriction fragement which contains the operator-promoter region and the nucleotide sequence coding for the amino-terminal region (first nine amino acids) of the lac z gene. In this case, protein fusions can be created between the ninth amino acid of β-galactosidase and the protein coded by the inserted gene. A series of vectors (pCΦI, pCΦII and pCΦIII) have been constructed in which unique EcoR1 sites have been positioned in the DNA such that protein fusions can be formed in all three reading frames (18) (Table III).

TABLE II

Commonly Used *Escherichia coli* Plasmid Cloning Vehicles

Plasmid	Replicon	Size (Kb)	Genetic Markers[a]	Unique Restriction Sites[b]	Reference
General use plasmids					
pAC105	ColE1	2.4	E1^{imm}	EcoRI	4
pACYC177	P15A	3.45	Ap^R, Km^R	PstI(Ap^R), HincII(Ap^R), BamHI, HindIII(Km^R), SmaI(Km^R), XhoI(Km^R)	17
pACYC184	P15A	3.9	Cm^R, Tc^R	EcoRI(Cm^R), HindIII(Tc^R), BamHI(Tc^R), SalI(Tc^R)	17
pBR313	pMB1	8.8	E1^{imm}, Ap^R, Tc^R	EcoRI, HindIII(Tc^R), BamHI(Tc^R), SalI(Tc^R), HpaI	13
pBR322	pMB1	4.4	Tc^R, Ap^R	EcoRI, HindIII(Tc^R), BamHI(Tc^R), SalI(Tc^R), PstI(Ap^R), PvuI(Ap^R), AvaI, PvuII, ClaI(Tc^R)	14
pBR324	pMB1	8.2	E1^{imm}, CP, Ap^R, Tc^R	EcoRI(CP), SmaI(CP), HindIII(Tc^R) SalI(Tc^R), BamHI(Tc^R)	12
pBR325	pMB1	5.4	Ap^R, Cm^R, TC^R	EcoRI(Cm^R), PstI(Ap^R), PvuI(Ap^R) HindIII(Tc^R), BamHI(Tc^R), SalI(Tc^R), AvaI	12
pBR327	pMB1	3.3	Tc^R, Ap^R	HindIII(Tc^R), BamHI(Tc^R), SalI(Tc^R), PvuI(Ap^R), PstI(Ap^R) AvaI, EcoRl	56
pBR328	pMB1	4.9	Tc^R, Ap^R, Cm^R	PvuII(Cm^R), BalI(Cm^R), EcoRl(Cm^R) HindIII(Tc^R), BamHI(Tc^R), SalI(Tc^R) PvuI(Ap^R), PstI(Ap^R), AvaI	56

ColE1	ColE1	6.4	$E1^{imm}$, CP	EcoRI(Cp), SmaI(CP)	35
pCR1	ColE1	11.2	$E1^{imm}$, Km^R	EcoRI, HindIII(Km^R)	22
pKB158	pMB1	2.4	Tc^R, λ^{imm}	BamHI(Tc^R), SalI(Tc^R), EcoRI, BglII, HpaI	6
pMB9	pMB1	5.3	$E1^{imm}$, Tc^R	EcoRI, HindIII(Tc^R), BamHI(Tc^R), SalI(Tc^R), HpaI, SmaI	13
pMK16	ColE1	4.5	Km^R, Tc^R, $E1^{imm}$	SalI(Tc^R), HincII(Tc^R), BamHI(Tc^R), SmaI(Km^R), XhoI(Km^R), EcoRI	37
pMK20	ColE1	4.1	Km^R, $E1^{imm}$	HindIII(Km^R), SmaI(Km^R), XhoI(Km^R), EcoRI, PstI	37
pMK2004	pMB1	5.2	Km^R, Tc^R, Ap^R	SmaI(Km^R), XhoI(Km^R), EcoRI, BamHI(Tc^R), SalI(Tc^R), PstI(Ap^R)	37
pMK2005	ColE1	6.9	$E1^{imm}$, $TrpE^+$	BglII($TrpE^+$), HpaI($TrpE^+$) SmaI, EcoRI, HindIII	37
pRK248	RK2	9.6	Tc^R	SalI(Tc^R), BglII, EcoRI	37
pRK353	R6K	11.0	$TrpE^+$	BamHI, EcoRI	37
pRK646	R6K	3.4	Ap^R	PstI(Ap^R), BamHI, BglII	37
pRK2501	RK2	11.1	Tc^R, Km^R	SalI(Tc^R), HindIII(Km^R) XhoI(Km^R), BglII, EcoRI	37
RSF2124	ColE1	11.2	Ap^R, $E1^{imm}$, CP	EcoRl(CP), SmaI(CP), BamHl	55
pSC101	SP219(?)	8.5	Tc^R	EcoRl, HindIII(Tc^R), BamHl(Tc^R), Sall(Tc^R)	21
pVH51 (mini ColE1)	ColE1	3.2	$E1^{imm}$	EcoRI	34

Plasmid	Replicon	Size (Kb)	Genetic Markers[a]	Unique Restriction Sites[b]	Reference
Low copy number plasmids					
pDF41	F'lac	12.8	$TrpE^{+}$	BamHI, EcoRI, HindIII, SalI	37

Heat inducible copy increase plasmids.

Plasmid	Replicon	Size (Kb)	Copy Number Uninduced/Induced	Genetic Markers[a]	Unique Restriction Sites[b]	Reference
pKC16	pBR322/λNOP	11.4	50/127	Ap^{R}	BamHI, SalGI	51
pKN402	R1drd19	6.9	25/>2000		HindIII, EcoRI	63
pKN410	R1drd19	15.2	25/>2000	Ap^{R}	HindIII, EcoRI, BamHI	63
pMOB45	pKN402	10.5	15/118	Cm^{R}, Tc^{R}	EcoR1(Cm^{R}), BamHI(Tc^{R}), HindIII(Tc^{R}), ClaI(Tc^{R})	10

[a] Abbreviations used for genetic markers are: Ap^{R}, ampicillin resistance; Cm^{R}, chloramphenicol resistance; CP, colicin production; Km^{R}, kanamycin resistance; Tc^{R}, tetracycline resistance; $E1^{imm}$, immunity to colicin E1; λ^{imm}, immunity to phage λ; $TrpE^{+}$, production of the protein product of the TrpE gene.

[b] When cloning at a restriction site insertionally inactivates a gene, that gene is indicated in parentheses.

TABLE III

Plasmid Vectors for Controlled Protein Expression

Plasmid	Replicon	Size (Kb)	Transformant[a] Selection	Promoter	Unique Restriction Sites for Insertion into Transcript	Number added to the amino terminus of a phase fused insert	Reference
pCΦ1	pBR322	3.4	Ap^R	lac	EcoRI	9	18
pCΦ2	pBR322	3.4	Ap^R	lac	EcoRI	9	18
pCΦ3	pBR322	3.4	Ap^R	lac	EcoRI	10	18
pHUB2	pMK2004	7.6	Km^R	λP_L	HpaI, EcoRI, BamHI, SalI	not given	9
pHUB1	pBR322	7.8	Tc^R	λP_L	Hpal, EcoRI, BamHII, SalI	not given	9a
pHUB4	pMK2004	6.5	Km^R	λP_L	HpaI, BamHI, SalI	not given	9
pMOB48	pKN402	9.5	Cm^R	lac	BamHI	8	10
pOP203	pMB9	5.3	Tc^R	lac	EcoRI	7	31
ptrpED5-1	pBR322	6.7	Ap^R	trp	HindIII	80(approximate)	32
pWT111	pBR322	4.8	Ap^R	trp	HindIII	9	59
pWT121	pBR322	4.8	Ap^R	trp	HindIII	11	59
pWT131	pBR322	4.8	Ap^R	trp	HindIII	13	59

[a]Abbreviations used are: Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Km^R, kanamycin resistance; Tc^R, tetracycline resistance.

TABLE IV
Plasmid Directed Overproduction of a Cloned Gene (β-galactosidase).[a]

	β-galactosidase Specific Activity[b]			
	Growth Conditions			
Strain	30°C	30°C + IPTG[c]	40°C	40°C + IPTG[c]
CR63 (mid-log phase)	72	8,164	74	13,990
JM101/pMOB53 (mid-log phase)	4,456	195,331	14,326	543,333
CR63 (lag phase)	44	6,250	73	21,655
JM101/pMOB53 (lag phase)	5,211	192,762	22,167	426,016

[a]A comparison of the expression of the β-galactosidase structural gene cloned into expression plasmid pMOB48 (plasmid pMOB53 in E. coli lac z^- strain JM101) with the normal expression of β-galactosidase from the chromosomal locus (lac z^+ E. coli strain CR63).

[b]The units given are manomoles o-nitrophenol produced per minute per mg protein at 25°C. Cell growth and measurement of enzyme activity are described in ref. 10.

[c]IPTG (isopropyl β-D-thiogalactopyranoside) is a gratuitous inducer of the lac operon.

Another lac promoter construct utilizes the modified 785 bp HindII fragment described by Messing and co-workers (45). This fragment contains a 42 bp synthetic oligonucleotide inserted at the nucleotide position corresponding to the fifth amino acid of β-galactosidase. The synthetic oligonucleotide, which contains a number of unique restriction sites, has been inserted so that 14 amino acids are added in frame to β-galactosidase. The NH_2-terminal β-galactosidase fragment synthesized is still active in α-complementation with an appropriate lac z deletion. Inserts in this region can be readily detected by the loss of α-complementing activity which can be assayed on X-gal plates.

This fragment has been inserted in a runaway replication mutant of R1drd19 (pMOB48) and can be used for the controlled expression of genes cloned in this vector (10). To test the efficiency of this system, a β-galactosidase gene lacking a promoter was cloned into the BamH1 site present in the 42 bp synthetic oligonucleotide (producing plasmid pMOB53). The effects of temperature and lac induction on β-galactosidase production directed by this plasmid were determined and the results are presented in Table IV. Under the conditions of temperature induction used in these experiments, the plasmid was present in about 120 copies per chromosome at 40°C compared to 15 copies at 30°C. Under these

same conditions, the specific activity of β-galactosidase increased from 4,456 units (uninduced, 30°C) to 543,333 units (40°C, induced). This is about a 40 fold increase in β-galactosidase levels compared to a fully induced culture containing a single copy of the wild type gene (see Table IV, strain CR63).

Vectors with TRp Operon Regulation. The *trp* operon consists of five structural genes and a regulatory region. Expression of these genes is controlled by a regulatory gene (*trp*R) which maps in a different region of the *E. coli* chromosome (for a review see 66). In the presence of excess tryptophan the affinity of the repressor for the operator region increases, preventing transcription. Expression of the operon can also be controlled after transcription has been initiated by a process referred to as attenuation (67). In attenuation, high levels of tryptophan lead to termination of transcription in the *trp* operon mRNA leader region before the structural genes are transcribed. The *trp* operon can be derepressed by mutations in the *trp*R gene or the operator-promoter region, by tryptophan limitation or by the addition of the tryptophan analog, 3β-indolylacrylic acid (49).

A number of controlled expression vectors based on the *trp* regulatory region have been constructed. In one series of vectors constructed by Tacon *et al*. (pWT111, pWT121 and pWT131) the operator-promoter region and the nucleotide sequence corresponding to the amino-terminal region of the *trp*E gene were inserted in pBR322 (59). These vectors allowed protein fusions to be constructed at the HindIII site in all three reading frames (Figure 2). Other *trp* vectors have been constructed which utilize the *trp*ED region (*trp* ED 5-1) and the *trp* leader region (ptrpL1) (32 and Hallewell, personal comm.).

Vectors With Lambda P_L Regulation. A number of vectors have been constructed which allow regulated expression of foreign DNA utilizing the P_L promoter of bacteriophage lambda. This promoter, which is regulated by the level of the lambda cI repressor and the product of the cro gene, can be conveniently controlled by utilizing a cI(ts), cro^- strain. In such strains, an increase in temperature leads to the inactivation of the repressor and, thus, high levels of transcription from the lambda P_L promoter (9).

Three vectors which contain the P_L promotor were constructed by Bernard *et al*. (9). These plasmids are derivatives of pBR322 (pHUB1) or pMK2004 (pHUB2 and pHUB4), pHUB1 and pHUB2 contain four unique restriction sites adjacent to the promoter (HpaI, EcoR1, BamH1 and SalI). pHUB 4 carries the lambda P_L promoter and N gene. Three unique restriction sites (HpaI, BamH1 and SalI) are present in pHUB4. By inserting the *trp*A gene in various sites in these vectors, it was demonstrated that under conditions of temperature

TABLE V

Plasmids for the Detection of E. coli RNA Polymerase Promoters and Terminators in Restriction Endonuclease Generated DNA Fragments

Plasmid	Replicon	Size (Kb)	Transformant Selector[a]	Phenotypic Change[b]	Unique Restriction Sites at Insertion Point	Reference
Promoter detector plasmids						
pBRH4	pBR322	4.4	Ap^R	$Tc^S \rightarrow Tc^R$	EcoRI	64
pGA24	p15A	6.1	Ap^R, Cm^R	$Tc^S \rightarrow Tc^R$	HindIII	2
pGA39	p15A	4.7	Cm^R	$Tc^S \rightarrow Tc^R$	HindIII, SmaI, PstI	2
pGA46	p15A	4.4	Cm^R	$Tc^S \rightarrow Tc^R$	HindIII, BglII, PstI	2
pKO-1	pBR322	3.9	Ap^R	$galK^- \rightarrow galK^+$	EcoRI, HindIII, SmaI	43
pMC1403	pBR322	9.9	Ap^R	$lac^- \rightarrow lac^+$	EcoRl, SmaI, BamHI	15
Terminator detector plasmids						
pBdCl	pBR322	5.3	Ap^R	$Tc^R \rightarrow Tc^S$	HindIII	23
pEP70	pBR345	7.2	$TrpE^+$	$Tc^R \rightarrow Tc^S$	PstI	28
pEP71	pBR345	7.2	$TrpE^+$	$Tc^R \rightarrow Tc^S$	KpnI	28
pEP165	pBR345	7.2	$TrpE^+$	$Tc^R \rightarrow Tc^S$	HindIII	28
pEP168	pBR345	7.2	$TrpE^+$	$Tc^R \rightarrow Tc^S$	EcoRI	28
pKG1800	pBR322	4.6	Ap^R	$GalK^+ \rightarrow GalK^-$	HindIII, SmaI	43
pMC81	pACYC999	25.6	Ap^R, $ColE1^{imm}$, $araC^+$	$lac^+ \rightarrow lac^-$	HindIII, KpnI	16

pMC279	pACYC999	11.0	Ap^R	$lac^+ \longrightarrow lac^-$	HindIII, SmaI, XhoI	16
pMC489	pACYC177	4.3	Km^R	$lac^+ \longrightarrow lac^-$	BamHI	16
pMC661	pACYC177	7.8	Km^R, $araC^+$	$lac^+ \longrightarrow lac^-$	HincII, HpaI	16
pMC692	pACYC177	5.0	Km^R	$lac^+ \longrightarrow lac^-$	EcoRI	16

[a] Transformants may be selected by: resistance to ampicillin (Ap^R); immunity to colicin E1 ($ColE1^{imm}$); resistance to chloramphenicol (Cm^R); resistance to kanamycin (Km^R); production of TrpE protein ($TrpE^+$); or production of araC protein ($araC^+$).

[b] Relative strengths of promoters or terminators may assessed by: change in sensitivity to tetracycline (Tc); changes in the specific activity of galactokinase (GalK); or changes in the specific activity of β-galactosidase (lac).

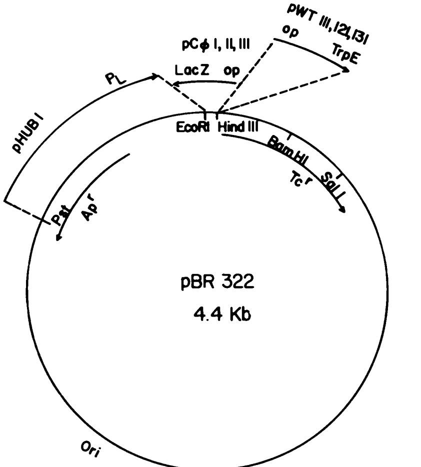

Figure 2. The structure of some protein expression vectors derived from pBR322. The promoter fragments used in the various constructs, as well as the region of insertion (or substitution) in pBR322 are indicated. The direction of transcription from the controlled promoters is indicated by the arrows.

induction, 1-6.6% of the total cell protein was the product of the trpA gene (9,9a).

Transcriptional Signal Detecting Vectors

Two specialized classes of plasmid vectors have been described which can be used for the detection of bacterial promoters and terminators (Table V).

Promoter detector vectors carry a gene without a promoter. By inserting a restriction fragment which carries a promoter upstream from the gene, expression of the gene can be activated. Expression can either be detected by the appearance of a resistance phenotype ($Tc^S \rightarrow Tc^R$) or by the use of sugar fermentation indicator plates ($lac^- \rightarrow lac^+$, or $gal^- \rightarrow gal^+$). By measuring the levels of enzyme produced, the strength of the promoter can be determined.

Plasmids have also been constructed which can detect transcription terminators. In these plasmids, insertion of a restriction fragment bearing a terminator at a restriction site located between a promoter and its structural gene leads to a decrease in expression of the gene. Again, indicator plates and enzyme assays are used to detect and quantitate termination signals.

SUMMARY

A wide variety of plasmid cloning vectors, most of which utilize the basic ColElI replicon have been constructed. Utilizing these vectors, in conjunction with the newly developed techniques of gene isolation and oligonucleotide systhesis, essentially any gene which can be identified can be cloned. We anticipate that future work in this area will be directed at improving techniques for the regulated expression of cloned genes and the further development of plasmid replicons in which the copy number can be readily controlled.

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EXPRESSION OF A FOREIGN PROCARYOTIC GENE IN *BACILLUS SUBTILIS*

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INTRODUCTION

Bacillus subtilis possesses the most thoroughly studied genetic system among gram-positive bacteria. Genetic analysis of this organism by means of transduction and transformation has lead to the development of a circular genetic map, the identification and approximate localization of the replication origin and terminus for the chromosome, as well as to studies of extrachromosomal inheritance (1,2). In recent years gene cloning in *B. subtilis* has been accomplished by several laboratories.

A major question that has arisen as the result of gene cloning studies in *B. subtilis* involves the inability to achieve expression of foreign genes, such as those of *E. coli*, in *B. subtilis*. Kreft *et al*. (3) constructed joint plasmids by ligating *E. coli* plasmids to plasmids capable of replicating in *B. subtilis*. Introduction of the joint plasmids into *E. coli* allowed expression of those drug resistance traits specified by both the *E. coli* plasmid and the *Bacillus* plasmid. However, introduction of such joint vectors into *B. subtilis* resulted in expression of only those drug resistance traits present on the *Bacillus* portion of the joint vector. Lee *et al*. (4) have demonstrated that *B. subtilis* RNA polymerase is significantly less active in initiating transcription from an *E. coli* promoter than is *E. coli* RNA polymerase. Therefore, we thought the inability to demonstrate expression of *E. coli* genes cloned in *B. subtilis* might be due to a lack of appropriate transcription. Accordingly, we chose to clone an *E. coli* gene into a segment of DNA that was known to be efficiently transcribed in *B. subtilis*.

In the present communication, we describe a uniquely constructed recombinant vector plasmid that permits the inducible expression of an *E. coli* gene in *B. subtilis*.

RESULTS

Construction of a Plasmid for Cloning DNA Fragments that Promote Gene Expression in *B. subtilis*

The approach taken involved identifying a DNA fragment containing a gene that was expressed in *B. subtilis* and provided an easily selected host trait. Secondly, it was necessary to make deletions from the fragment which resulted in loss of gene expression but left the structural gene intact. pPL531 (Fig. 1) contains a 1.44 Md *Eco*R1 DNA fragment cloned in pUB110 from *B. pumilus* chromosome digests. This fragment contains a structural gene specifying a chloramphenicol (Chr) inducible chloramphenicol acetyltransferase (CAT). The vector plasmid, pUB110, is a small (3 Md) high copy plasmid that specifies neomycin resistance in *B. subtilis*.

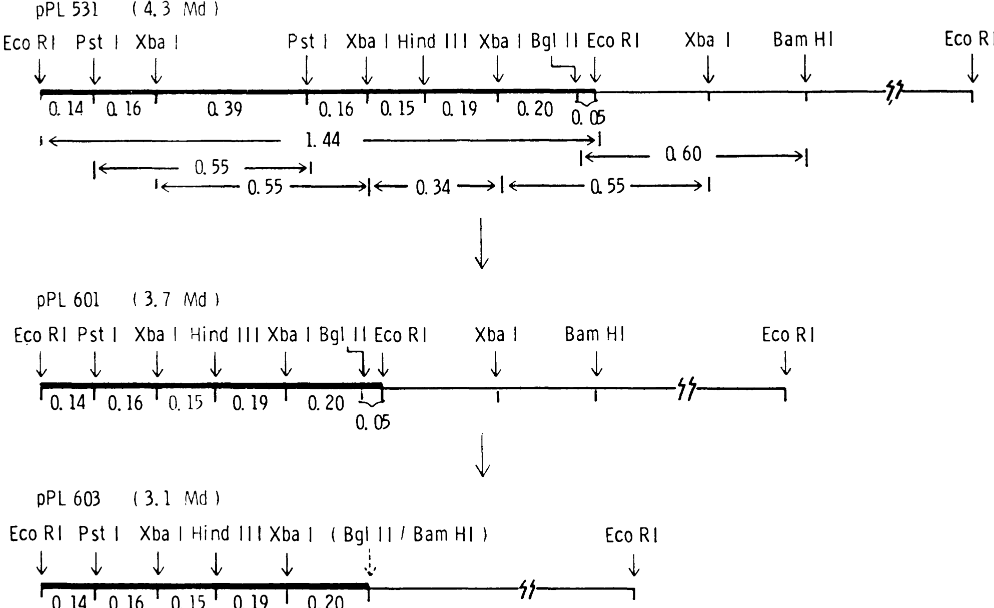

Figure 1. Restriction endonuclease maps of pPL531, pPL601 and pPL603. The thickened horizontal line indicates the cloned *B. pumilus* DNA. Taken from ref. 5.

The unique Hind III site in pPL531 lies within the cloned fragment and DNA inserts made at this site inactivate CAT. These data suggested that the Hind III site marked the location of the CAT gene. Deletion of the Pst-1 fragment from the cloned CAT fragment resulted in a derivative of pPL531, pPL601, that no longer specified high level chloramphenicol resistance in B. subtilis (Fig.1, ref. 5). However, reversal of the orientation of the EcoR1 fragment in pPL601, yielding pPL602, restored the ability of the plasmid to confer resistance to high levels of this antibiotic (5). Therefore, it was evident that the Pst-1 deletion from pPL531 removed a region necessary for expression of the CAT gene, but that the CAT structural gene remained intact. The EcoR1 site in pPL601 distal to the Pst-1 site was removed by a BamH1 + Bgl II cleavage and ligation producing pPL603 (Fig. 1). EcoR1 and EcoR1* fragments of Bacillus DNA were cloned into this site to identify those fragments which restored high level CAT gene expression on pPL603. pPL608 is one such derivative of pPL603 and contains a 0.2 Md EcoR1* promoter fragment. The specific activity of CAT specified by pPL608 is about 10-fold higher than that specified by the parent plasmid, pPL531 (5).

Expression of E. coli trp Genes Cloned in B. subtilis

We chose to insert a segment of DNA not normally expressed in B. subtilis into the Hind III site of pPL608. The E. coli DNA segment selected was that spanning the trp C(F) gene (6). This was our choice in part because E. coli trpC(F) resides on a Hind III fragment, and because our previous work with Bacillus trp segments cloned in B. subtilis indicated that even low level of expression of cloned trpC was sufficient to complement the trpC2 mutation in B. subtilis (7,8). The 1.7 Md Hind III fragment spanning E. coli trpC(F) was cloned from plasmid pVH5 (9) and from chromosome digests of E. coli HB101. The trp fragment cloned from both sources appeared functionally identical. pPL608-5 is an example of a derivative of pPL608 that contained a trp fragment cloned from pVH5 (Fig. 2).

pPL608-5 complemented point mutations in B. subtilis trp D, C and F genes, but did not complement mutations in trpE, B and A. Removal of the trp insert, by Hind III cleavage, restored CAT activity specified by the parent plasmid and deleted trpD, C, F complementing activity. The 1.7 Md insert in pPL608-5 comigrated with a Hind III digest product of pVH5 agarose gel electrophoresis. Moreover, nick translated pVH5 hybridized to the 1.7 Md insert in pPL608-5 (data not shown). Removal of the promoter from pPL608-5, by EcoR1 cleavage, eliminated the ability of the plasmid to complement trp mutations in B. subtilis, as did reversal of the orientation of the Hind III insert. Thus, sequences present in the vector are essential to the expression of the cloned E. coli genes.

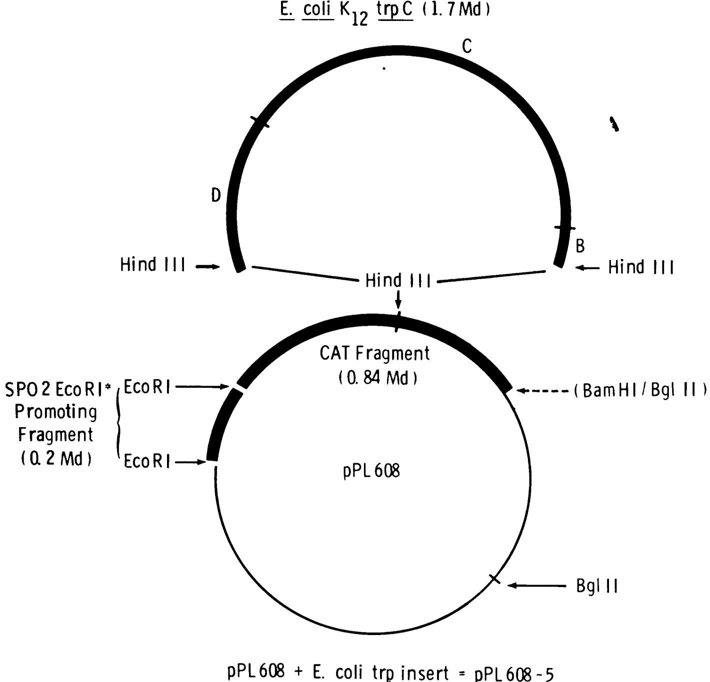

Figure 2. Diagram of the insertion and orientation of the 1.7 Md *E. coli* trpC fragment in pPL608-5.

Chloramphenicol Inducible Expression of Cloned *E. coli trpC*.

The *E. coli trp* fragment was inserted into pPL608 at the *Hind* III site which likely lies within the structural gene for CAT. Since the CAT gene behaves as though its expression is inducible by Chr, we tested the possibility that the inserted *trp* genes might also be Chr inducible. *B. subtilis* harboring pPL608-5 was grown to mid-log phase, and the culture was split. To one was added a subinhibitory concentration of Chr (0.1 μg/ml). During the subsequent 100 min of growth the Chr treated culture showed a 7-fold increase in the specific activity of the *trpC* gene product, indol glycerolphosphate synthetase, whereas the untreated culture showed no increase in this enzyme (Fig. 3). In contrast to the apparent regulation by Chr, we found no change in the specific activity of the *trpC* gene product by varying the level of tryptophan in the growth medium.

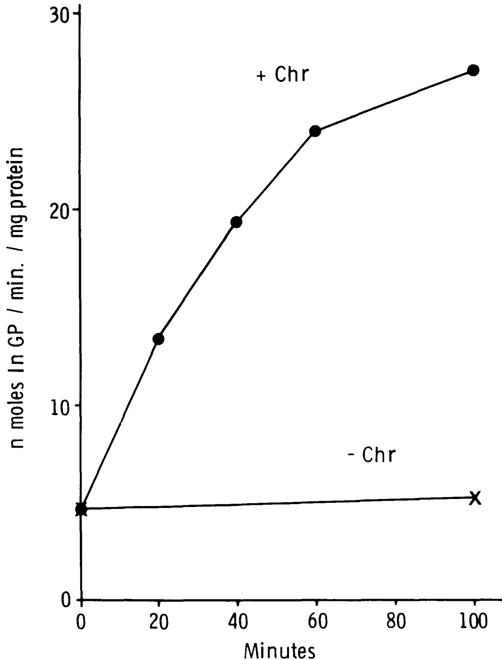

Figure 3. Induction by chloramphenicol of InGPS specified by pPL608-5. BD224 (pPL608-5) was grown to the middle of the exponential growth phase in Min CH containing 100 μg per ml of tryptophan. The culture was split and 50 ml samples were withdrawn from each. A subinhibitory concentration of Chr (0.1 μg/ml) was added to one culture, and during subsequent growth, 50 ml samples were periodically withdrawn. Cells in each sample were harvested, processed and assayed for InGPS as previously described (8,10).

CONCLUSION

The 1.7 Md *Hind* III *E. coli trp* fragment cloned in pPL608 was identified by complementation of a *trpC* mutation in the recipient, *B. subtilis*. The cloned fragment was subsequently shown to complement mutations in the *B. subtilis trp D*, *C* and *F* genes. In the *E. coli trp* operon two *Hind* III sites flank the *trpC(F)* gene. One is located in the beginning of *trpB* and the other is within *trp(G)D* (11, 12, 13). The *E. coli* genes *trpC(F)* and *trp(G)D* each specify proteins that have two activities. In *B. subtilis* each of these separate activities is specified by a gene; *trpC*, *trpF*, *trpG*, *trpD* (6). The cloned *E. coli trp* fragment contains that portion of the *trp(G)D* gene corresponding to the *trpD* gene product (12). Since this cloned fragment complements a *B. subtilis trpD* mutation,

the cloned trpD gene is being expressed. It is not known if the translation start codon for trpD expression resides in the cloned fragment, or if this start signal exists in the vector.

Expression of the cloned E. coli trp genes in pPL608-5 was shown to be dependent on the promoter fragment present in the vector. We suspect the promoter fragment provides sequences necessary for transcription initiation and that transcription occurs from the promoting fragment through the CAT structural gene. It will be useful to determine the range of foreign genes that can be expressed in B. subtilis when inserted into pPL608 and the changes in gene expression that result when different promoter fragments are examined.

ACKNOWLEDGEMENTS

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DISCUSSION

Q. HELINSKI: I was somewhat puzzled by the last construction and the cm induction of trpC expression. Isn't the CAT promotor absent in this construct? If so, would this suggest that chloramphenicol is inducing at the level of mRNA stabilization or at the translation level?

A. LOVETT: Insertion of different promoting fragments into pPL603 does not alter the inducivility of the CAT gene. Thus, the inducibility of CAT (or trp) by chloramphenicol is not a function of the promoting fragments. One possibility is that induction is at the translational level. This pint is under study.

Q. JOHNSON: Do different promotors produce different amounts of CAT activity?

A. LOVETT: The different promoting fragments we have cloned in pPL603 all promote similar levels of expression of the CAT gene, as measured by the specific activity of CAT.

Q. VAPNEK: Have you measured the levels of galactokinase in pK01 derivatives that carry B. subtilis promoters? Is a strong promoter in B. subtilis as strong in E. coli ?

A. LOVETT: We have not measured the levels of galactokinase.

FUSION OF MICROBIAL PROTOPLASTS: PROBLEMS AND PERSPECTIVES

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Induced fusion of protoplasts is a relatively new way of genetic manipulation in microbial systems. The individual steps of the manipulation are shown in the scheme of Figure 1. The rigid cell wall of bacteria, fungi, yeast, and algae (as well as of plant cells) can be removed without influencing the viability and integrity of the cell. The wall-less microorganism is surrounded by a plasma membrane, in hypertonic media it is spherical in shape, and is called a protoplast. The protoplasts, under appropriate conditions, do resynthesize cell wall material and revert to the original form with characteristic morphology.

Why then could not protoplasts be induced for fusion, reverted to the microbial form, and in that way produce genetic hybrids? The advantage of this type of manipulation would be enormous if it is taken into consideration that protoplast formation from any microorganism can be achieved (or at least there are no data contradicting the possibility). So, any microorganism lacking mechanisms for genetic information transfer between cells could be artificially made "sexual". The field does not yet count more than about two hundred published papers including the use of bacteria, fungi, yeast, and algae. A recent review by Ferenczy (8) is quite a complete historical account of the publications to the middle of the year 1980. (The history of this paper, therefore, is condensed to the minimum and appears in Table I, parts A,B,C.)

The magic agent incuding fusion of protoplasts was found by Kao and Michayluk (21) in 1974. They demonstrated that polyethylene glycol (PEG) is highly active in inducing fusion of plant protoplasts. Since then, the application of PEG in any microbial system tested has also proved to be successful for inducing fusion of protoplasts.

TABLE I

A. Backgrounds for Comlementation in Prokaryotes

Formation of Transient Diploids and/or Near-Diploids, and Stable Haploid Recombinants

Gram-Positive Bacteria

Bacillus megaterium (Fodor and Alföldi, 1976, 1979).
Bacillus subtilis (Schaeffer *et al.*, 1976, Levi *et al.*, 1977, Hotchkiss and Gabor, 1980).
Brevibacterium flavum (Kaneko and Sakaguchi, 1979).
Staphylococcus aureus (Hirachi *et al.*, 1979).
Streptococcus lactis (Gasson, 1980).
Bacillus thuringiensis (Grigorieva and Azizbekian, 1980).

Gram-Negative Bacteria

Escherichia coli (Tsenin *et al.*, 1978).
Providencia alcalifaciens (Coetzee *et al.*, 1979).

Actinomycetales

Streptomyces coelicolor (Hopwood *et al.*, 1977, 1979).
S. parvulus, *S. lividans* (Hopwood and Wright, 1978, 1979).
S. griseus, *S. acrymicini*
S. fradiae, *S. griseofuscus* (Baltz, 1978).
S. fradiae, (+) *S. bikiniensis* (Godfrey *et al.*, 1978).
S. parvulus, *S. antibioticus* (Ochi *et al.*, 1979).

Micromonospora echniospora (Szvoboda *et al.*, 1980).
M. inyoensis

B. Backgrounds for Intraspecies Complementation in Eukaryotes

Heterokaryosis: *Geotrichum candidum* (Ferenczy *et al.*, 1972, 1974), *Phycomyces blakesleanus* (Binding and Weber, 1974), *Mucor racemosus* (Genther and Borgia, 1978).

Heterokaryosis with Occasional Diploid (and Aneuploid) Formation: *Aspergillus nidulans* and other Aspergilli (Ferenczy *et al.*, 1974, 1975, 1976), *Penicillium chrysogenum* and other Penicillia (Anné and Peberdy, 1975, 1976, Peberdy *et al.*, 1977, Pesti *et al*, 1980).

Heterokaryosis with Frequent Diploid Formation: *Candida tropicalis* (Fournier *et al.*, 1977, Ferenczy *et al.*, 1977, Vallin and Ferenczy, 1978), *Candida albicans* (Sarachet *et al.*, 1981, Pesti and Ferenczy, 1981).

(Table I, Part B, continued)

Heterokaryosis with Transient Diploids and Haploid Recombinants
Cephalosporium acremonium (Hamlyn and Ball, 1979), Trichoderma reesi (Manczinger and Ferenczy, 1981).

Diploid Formation (Unstable): Saccharomycopsis lipolytica (Stahl, 1978).

Diploid Formation (Stable): Schizosaccharomyces pombe (Sipicki and Ferenczy, 1976, 1977), Saccharomyces cerevisiae (Ferenczy and Maraz, 1977, Solingen and Plaat, 1977, Svoboda, 1977, Yamamoto and Fukui, 1977), Rhodosporidium toruloides (Sipiczki and Ferenczy, 1977), Kluyveromyces lactis (Morgan et al., 1977), Chlamydomonas reinhardtii (Matagne et al ., 1979), Pichia guillermondii (Bottcher et al., 1980), Hansenula polymorpha (Savchenko and Kapulsevich, 1980).

Polypoloid Formation: Saccharomyces cerevisiae (Takano and Arima, 1979).

C. Backgrounds for Interspecies Complementation in Eukaryotes

Heterokaryosis with Occasional Diploid (and Aneuploid) Formation:

Penicillium chrysogenum + P. notatum (Anné and Peberdy, 1975, 1976).
Penicillium chrysogenum + P. cyaneo-fulvum (Peberdy et al., 1977).
Penicillium citrinum + P. cyaneofulvum (Anné and Eyssen, 1978).
Aspergillus nidulans + A. rugulosus (Kevei and Peberdy, 1977, 1979).

Formation of "Interploids" (= "Interspecific Heteroploids" = "Partial Alloploids" = "Interspecific Aneuploids"):

Penicillium roquefortii + P. chrysogenum (Anné et al., 1976).
Aspergillus nidulans + A. fumigatus (Ferenczy, 1976, Ferenczy et al., 1977).
Schizosaccharomyces pombe + S. octosporus (Sipiczki, 1980).
Candida albicans + C. tropicalis (Kucsera and Ferenczy, 1981).

The list of microorganisms for which PEG induced fusion of protoplasts has been hitherto demonstrated and is given in Table I, parts A,B,C. The possibility of constructing inter-species models as listed in Table IC should also be emphasized.

The already accumulated data are quite impressive. Nevertheless, inducing fusion of microbial protoplasts in some microbial systems seems to be rather an art than a science. If we examine the

procedure in detail, it will be immediately clear upon what this statement is based.

Since, in this presentation I am going to point to the problems of the system, I do not discuss the formation of protoplasts and their stabilization. For example, removal of the rigid cell wall in the great majority of cases can be achieved by enzymatic digestion, under well defined conditions (which does not mean that those conditions are always easily found). The right osmolarity, ionic balance and pH are the key factors not only for the formation of protoplasts but also for their stabilization.

When we have the stable protoplasts we may start wondering about the reversion to microbial form, what is going on, and how the events are regulated. Namely, it should be kept in mind that in each system of microbial protoplast fusion, as a result, microbial forms are sought, obtained, and analyzed.

The reversion of microbial form should be understood as follows: a.) How the cell wall is resynthesized; b.) How the characteristic microbial form is regained; c.) What are the regulatory mechanisms behind these events?

Not a single system exists yet where all these questions could be answered, but there are many where none of them has yet been even raised. As a matter of fact, often one is interested only to know how many individual protoplasts of a suspension (or what percentage of them) are capable of yielding the bacillary form on "reversion medium" specified for the given microorganism.

Really, under right conditions, it is possible to regularly obtain 100% regeneration from protoplasts, as did Gabor and Hotchkiss (13) working with B. subtilis and Baltz with Streptomyces griseofuseus (1a). Working with Streptomyces parvulus, Ochi, Hitchcock, and Katz (25) developed a chemically defined medium where the efficiency of regeneration of protoplasts to mycelial forms was greater than 50%. At the other extreme, protoplasts of the Micromonospora reverted to microbial form only in the range of 10^{-3}, although this system can also be used for fusion experiments (29).

Polyethylene glycol does precipitate the protoplasts into huge random aggregates, which can be seen in some systems even with the naked eye. Nevertheless, after having removed the PEG, the aggregates dissociate in fresh medium, liberating unharmed protoplasts, as far as their ability to revert to microbial form is concerned. Electronmicroscopic studies clearly visualized that in the aggregates multiple fusion events are quite common features (12). Genetic evidences will be mentioned later which show that from appropriate crosses via protoplast fusion multiparental recombinants can also be obtained (19).

A phage complementation test (26) revealed that the fusion event in that system may be as high as 50% of the estimated maximum. In the experiment, two strains of Bacillus subtilis, each lysogenic for a different Sus mutant of the phage ϕ 105, were induced by mitomycin-C, then protoplasted, treated with PEG and plated with ϕ 105 sensitive indicator bacteria. The frequency of the heterozygous fused cells could then be determined from the number of infectious centers produced.

So the fusion of protoplasts which seems to be a physico-chemical process certainly can be induced with a high frequency. What happens then within the fused protoplasts? All of the evidences show that the fused protoplasts remain metabolically active for a while.

Magda Gabor and Rollin Hotchkiss (13), studying the parameters governing bacterial regeneration after PEG treatment of protoplasts of Bacillus subtilis, demonstrated that some 80 to 90% of them are able to regenerate their cell walls and form colonies under the conditions specified. The ability may, however, be easily lost by a slight change of conditions. Furthermore, a specific class of their fusion hybrids, the biparentals (will be discussed in detail later), may be as large as 10% of the colony formers. This number is near or in the range of the physical fusion events. It might be expected, therefore, that under the right conditions, whatever they are, fused protoplasts should have the potential of reverting to microbial form. It might be expected also that the fused protoplasts would be extremely sensitive to metabolic and environmental influences. It would, therefore, be important to know the factors which determine or influence the fate of a fusion product.

Indeed, extreme sensitivity of the fused complex can be deduced from genetic data obtained with B. megaterium (11). The data show how delicate the system really is, since composition of the medium in which the bacteria were grown before the fusion strongly influences the distribution of recombinant classes on the different selection media. The phage complementation test of Sanchez-Rivas and Garro (26) also revealed that the composition of the growth medium affects the ability of protoplasts to fuse and to regenerate a cell wall.

Gabor and Hotchkiss (13), working with B. subtilis, deduced from their experiments that regeneration of recombinant-forming cells is independently determined and not closely related to the average regeneration for the population. Since different pregrowth conditions of the same bacterium, varying only in a few amino acids, already significantly alter the susceptibility of the recombinant forming units to regeneration, it is possible that cytoplasmic interactions between the parental components might be one of the factors involved (11).

Genetics via Fusion of Protoplasts. There are a few conclusions which already can be generalized from the data accumulated on fusion of microbial protoplasts:

a.) as to fusion itself, any protoplast can be fused with and other protoplast; there seems to be no genetic barrier or incompatibility at this level;
b.) mating type polarity does not play any role in the PEG induced fusion of protoplasts;
c.) true genetic recombinant microorganisms can be obtained within each species tested;
d.) in the formation of recombinants the parents seem to play an equal role;
e.) with eucaryotic microorganisms heterokaryons, synkaryons, diploids, polyploids, or true recombinants might respectively be formed after the fusogenic treatment;
f.) under carefully selected conditions, very high yields of recombinants can be achieved;
g.) the fusion technique can be successfully applied for genetic analysis even in systems offering no natural way to do it.

Nevertheless, it should be emphasized here that in this early period of research in this field each laboratory's main activity has been to establish the system of its interest, to find adequate proofs for the feasibility of the system and only in a very limited number to use it for some form of detailed genetic analysis.

It may be asked, therefore, what are the prospectives for the fusion of microbial protoplasts in genetic analysis? It is quite clear that much research has to be done to specify the conditions where regeneration of the fused protoplasts will be optimal for each system. But, even without such detailed studies on the reversion process, genetic studies are underway in many labortatories. In these experiments, microbial forms are studied either after having been regenerated on nonselective or on selective media. From the point of view of a genetic analysis both procedures may present certain drawbacks. On a nonselective media the genomes of the parents may segregate out before recombination takes place. By contrast, growth on a selective medium may provide an opportunity for an increased frequency of the recombination events.

High frequencies of apparent recombinants were observed with *Streptomyces parvulus* and *S. antibioticus*, respectively, by Ochi, Hitchcock, and Katz (25) which were three or more orders higher in magnitude than obtained by mating.

Baltz (1a) already has constructed a linkage map of *S. fradiae* using the protoplast fusion technique. Analysis of fusions between protoplasts of four different genotypes of *S. coelicolor* by Hopwood

and Wright (19) indicated that the crossing over between them produced recombinants inheriting markers from three or four parents. They postulated that complete genomes of the fused protoplasts might become fragmented and that crossing over, often repeated, might occur between the fragments to generate haploid recombinants.

The genetic analysis of the vegetative incompatibility system in *Aspergillus nidulans* by Dales and Croft (5) show how a problem can be studied only by a protoplast fusion approach even where a microorganism has natural ways for genetic information transfer. The vegetative or heterokaryon incompatibility system in *A. nidulans* precludes any further stages of parasexual development in this well known ascomycete. This leaves the enigma that the most desirable methods for studying the genetic control of the heterokaryon incompatibility system in *A. nidulans* would be by the observation of heterokaryon formation and by parasexual analysis of subsequent diploid or aneuploid strains derived from heterokaryon incompatible parents, though under normal circumstances this approach is prevented by the incompatibility system itself. Dales and Croft have demonstrated that it was possible to isolate heterokaryons and probable diploids from vegetatively incompatible parents using a protoplast fusion technique and this opened the way for conducting parasexual analysis of this problem.

Fused protoplasts of *Saccharomyces cerevisiae* from two different haploid strains with identical mating type in the experiments of Gunge and Tamaru (15) proceeded by karyogamy producing stable diploids of *aa* mating type. Crossed with opposite mating type diploids by the conventional method, sporulation of the resulting hybrid colonies, yielded the predicted results in tetrad analysis. Lückemann, Sipicki, and Wole (24), working with *Schizosaccharomyces pombe*, found that transmission and recombination frequencies of mitochondrial markers are similar in diploid clones derived from zygotes or from fusion of haploid protoplasts of identical mating type.

More data could also be quoted which seem to indicate that genetic analysis via protoplast fusion of eucaryotic microorganisms does not show anomalies, and does not raise special problems.

Nevertheless, one has to be careful in generalizing this conclusion, since there are data which show the contrary; Böttcher and his his colleagues (3), for example, studying genetic structure of yeast hybrids constructed by protoplast fusion, have noticed that among other anomalies the frequency of aberrant tetrads is significantly higher in the fusion than in the sexual hybrids.

In any case, it should be recalled in this respect that in eucaryotic microorganisms, somatic syncytium formation, heterokaryosis, and karyogamy are natural events as well as mitotic or meiotic segregation. All the mechanisms for these events are evolutionally present. The fusion of protoplasts within a single species therefore does not create an unnatural situation for the cell.

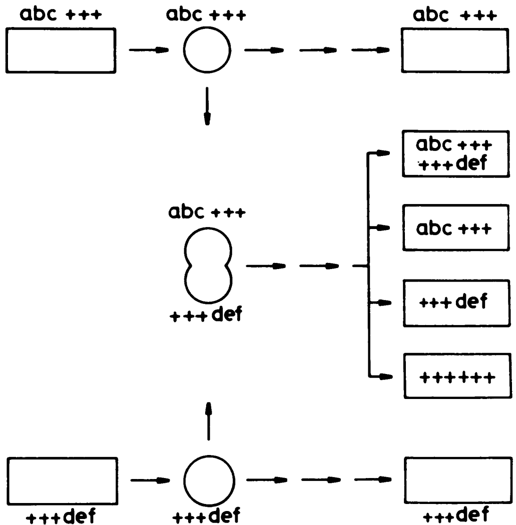

Figure 1. Scheme for explanation of fusion of microbial protoplasts.

The situation, however, seems to be more complicated in the eubacteria. We do not know any natural stage of the bacterial life cycle where cytoplasmic and whole genome interaction between individual cells would be involved. It is therefore impossible to predict what kind of bacteria will be obtained from a protoplast fusion.

It became evident in our very first experiments with fusion of protoplasts of B. megaterium that the progeny of a colony, obtained on a selective medium where neither of the parents can grow, in subculture may segregate individual bacteria with quite varied phenotypes and genotypes (10).

The natural ploidy level in the prokaryotic microorganisms is haploid. Lévi-Meyreuis, Sanchez-Rivas, and Schaeffer (23) have found that a minority among the prototrophic clones formed when protoplasts from two polyauxotrophic strains of Bacillus subtilis were fused were diploids, carrying the parental deficient alleles, and segregating auxotrophs when grown in rich medium. The same results were obtained in Rec^+ or Rec^- bacteria.

Something unprecidented in microbial genetics has been observed

by Hotchkiss and Gabor (20) working with multiple auxotrophic strains of B. subtilis. In their experiments they have plated PEG-treated protoplasts of triple auxotrophic strains (complementary to each other) on rich media under conditions in which the reversion of bacillary form is extremely high. Well separated regenerated colonies from as high as 10^{-6}, 10^{-5} dilutions were picked up at random and plated on non-supplemented or on supplemented media selective for either of the parents, respectively. Besides the colonies with expected phenotype they found a class of colonies which did not grow on non-supplemented medium but did grow on both parental media. These colonies were termed "biparental" (BP) by Hotchkiss and Gabor. When such biparental colonies were subcultured on a medium selective for one of the parents, and then tested for auxotrophy, in phenotype they seemed to be characteristic for that one of the parents which was expected to grow on the given medium, and were independent of those nutritional factors needed by the other parent. Yet after segregation from many of them, bacteria with characteristic nutritional requirement of the other parent did reappear. So, the biparentals (taking into consideration all the controls) might be diploids. But, if they were diploids, how is it possible that the biparental bacteria exhibit the phenotype of only one of the parents? Namely, the auxotrophy of the parents was complementary to each other, therefore, they ought to be complementing and to be phenotypically prototrophs. Thus, the biparentals discovered by Hotchkiss and Gabor are diploids though they are not complementing prototrophs. They show precisely the phenotype of either one of the parent strains. In other words, one important product of that protoplast fusion is a diploid bacterium carrying two complete genomes, only one of which is expressed in each particular clone, and the other will be manifested only at or about the time of segregation.

It is needless to say that if this kind of situation proves to be a general phenomenon, it would profoundly alter our view about the regulation of gene expression in bacteria. It would also be impossible to do complementation studies under such conditions.

Interestingly enough, Dancer and Mandelstam (6) have recently shown that protoplast fusion provides a simple means for studying complementation of sporulation mutants of B. subtilis. Nevertheless, it should be emphasized that the two lines complementing and non-complementing types of diploids do not contradict each other since the conditions for the experiments were completely different, which might have determined the fate of the fused protoplasts in opposite directions.

Practical Applications. The fusion of microbial protoplasts may offer for the industrial microbiologists a long awaited technique for genetic engineering on a cellular level. There are

already a few publications demonstrating the utility of induced fusion of protoplasts for the genetic manipulation of eucaryotic microorganisms of industrial importance.

Fermentation with conventional brewing yeast produces a beer in which remains a non-fermentable polysaccharide material conveniently termed 'dextrin'. Hockney and Freeman (18) reported the successful construction via protoplast fusion of a stable hybrid between Saccharomyces carlsbergensis (industrial strain) and Saccharomyces diastaticus which would produce acceptably flavored beer containing a reduced concentration of dextrin. The ability to utilize dextrin is a character from the S. diastaticus, the strain which can not be used in industrial fermentation since it produces unacceptable flavor compounds in beer. Spencer, Land, and Spencer (28) recommended the use of mitochondrial markers to isolate hybrids by fusion of protoplasts from some brewing and distiller's yeast.

The members of Actinomycetales (Streptomyces, Micromonospora, Actinomyces, Nocardia, etc.) play a key role in industrial antibiotic production, Intensive studies are underway to use the protoplast fusion technique for strain improvement or for construction of antibiotic producers with new specificity. Published data refer to Streptomyces coelicolor, S. parvulus, S. lividans, S. griseus by Hapwood et al. (19a); to S. fradiae and S. griseofuseus by Baltz (1a); S. parvulus and S. antibioticus by Ochi, Hitchcock, and Katz (25); Micromonospora echinospora and M. inyoensis by Szvoboda et al. (29). Interspecies hybrid construction between Streptomyces fradiae and S. bikiniensis was attempted by Godfrey, Ford, and Huber (14).

Filamentous fungi are the other great antibiotic producers in the microbial world (Penicillium, Aspergillus, Cephalosporium, etc.). There is an intensive research on the inter-species hybridization via protoplast fusion with filamentous fungi, and many such hybrids are already described (see the review of Ferenczy (8)). All those interspecies hybrids constructed via protoplast fusion seem to be microorganisms harboring one or a few chromosomes from one of the parents in the complete chromosomal set of the other.

So, it seems as though the fusion of protoplasts could be used in a great variety of microorganisms for genetic manipulation on a cellular level.

Addendum. The technique of PEG-induced fusion of microbial protoplasts became the background for a few other techniques introduced into microbial genetics. A direct extension of the protoplast fusion is the selective organelle transfer (8) (mitochondria, chloroplast). During protoplast formation in budding yeast, small protoplasts are frequently released from the buds, containing only

mitochondria. These anulcleate small protoplasts can be easily separated and fused with normal protoplasts (9). Isolated mitochondria (16), isolated photosynthetic organelles of cyanobacteria or green algae (22), as well as plant chloroplasts (30) can also be taken up by fungal and yeast protoplasts after PEG treatment. Furthermore, not only organelles but isolated plasmid DNA uptake can be induced by PEG treatment of the microbial protoplasts (*Streptomyces* (2); bacteria (4,31); yeast (17)).

SUMMARY

Polyethylene glycol-(PEG)-induced fusion of both procaryotic and eucaryotic microorganisms seems to be a good technique for constructing hybrid microorganisms. Though the data published show that the fusion technique can be successfully applied for genetic analysis even in systems having no natural way to do it, further studies are needed to find, for each system, the conditions where it works in predictable ways.

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Table I (Parts A,B,C) was completed by L. Ferenczy in 1981 and has been used here with his kind permission. The cited literature can be found in his review.

STRATEGIES: MODELS AND PROBLEMS

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This afternoon we shall continue to discuss methods of modifying DNA in microorganisms with the object of increasing their economic value to man. But before any engineering is begun, we might take note of microbial enzymatic reactions at present in operation. What general patterns have been established by the more subtle pressures of evolution? Time is not available for an adequate survey, and a few examples from one important area must suffice: these will be taken from the reactions employed by aerobic microorganisms to degrade aromatic compounds. As a unit of biochemical structure, the benzene nucleus takes second place only to glucosyl units, and there is more lignin to be found in nature than protein. However, man, rats and *E. coli* have very little ability to degrade aromatic compounds, although it may be noted that recent work has shown that some strains of *E. coli* exhibit greater expertise than was once believed. And since these species have received so much attention, many biochemists who turn their attention to soil isolates find that bacterial aromatic catabolism is complicated and confusing. It is, in fact, neither; because the never-ending degradation of the Earth's aromatic natural products has been confined to eight or so separate and distinct metabolic channels leading into the Krebs cycle. Separation of pathways is assured by the specificities of enzymes for their substrates and by corresponding narrow specificities of derepression mechanisms. Similar properties are shown by bacterial monooxygenases that prepare aromatic acids for entry into the sequences: these are flavoproteins, contrasting sharply with the cytochrome P-450 systems of liver which hydroxylate so many xenobiotics without discrimination. There is no such enzyme as an all-purpose bacterial aromatic oxygenase. David Gibson's group has shown that hydroxylation of aromatic hydrocarbons by filamentous fungi apparently is a less specific operation, but like

that of the liver, the enzyme system functions to serve the ends of detoxication and excretion. In bacteria, hydroxylation prepares the molecule for degradation and assimilation by a carefully selected route to the Krebs cycle.

When the various reaction sequences for degrading aromatic natural products were known, it became possible to predict those for certain man-made compounds. This fact has not always been appreciated, and there have been occasions when perfectly predictable patterns of catabolism have been rediscovered and published. Two points may be made in this connection. Our own experiments with the versatile eukaryote, *Trichosporon cutaneum* revealed several variations upon established prokaryotic themes; but the central catabolic strategy - based upon the biochemistry of specific oxygenases - was not modified. And second, while the "real world" of nature is much more complicated than that of the laboratory, there are few observations made with mixed substrates and cultures that cannot be interpreted in the light of principles established for simple systems.

BAKER'S YEAST: A SUCCESSFUL INDUSTRIAL MICROORGANISM IS NOW A FAVORABLE HOST FOR MOLECULAR CLONING

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INTRODUCTION

The fact that baking and brewing yeasts are successful industrial microorganisms is without question. They have been used for centuries for the production to carbon dioxide and ethanol in their respective applications, and more recently have been used as a source for naturally contained vitamins, enzymes and cofactors of commercial and scientific value (1). Since their adaptation to the genetics laboratory in the 1930s and 40's by Winge, Lindegren and collaborators (2,3) in the form of cooperative strains of Saccharomyces cerevisiae, baker's yeast has become a very well developed eukaryotic genetic system, and one which is most amenable to biochemical fractionation and analysis.

With the advent of molecular cloning techniques, yeast first became a source of interesting eukaryotic genes which could be expressed in Escherichia coli (4-7). Later, with the development of transformation procedures to return DNA to yeast (8,9), it became highly desirable as a host for molecular cloning experiments. Early applications involved investigation of the expression of yeast genes when returned to yeast, either as single copy chromosomal integrants, or as multicopy autonomous plasmids (7,19). Soon thereafter, yeast was used as a host for expression of genes and other functional sequences from other organisms (10). The combination of yeast's traditional genetic and biochemical facility, and the new molecular cloning technology, has resulted in very rapid development of new yeast vectors, hosts, and strategies of application of the system to both basic research problems and the needs of industry. This paper will summarize the types of vectors and host strains

presently available, and will then discuss strategies of application of the system, some of which are currently in use, and others which are at present only proposed.

YEAST STRAINS SUITABLE AS HOSTS FOR MOLECULAR CLONING

In principle, any common laboratory strain of *S. cerevisiae*, into which a genetic lesion can be introduced to be used as a selection marker, is a suitable host for molecular cloning experiments. In practice, however, some strains are more efficiently transformed than others, and some may have additional features which make them more suitable than others for particular applications. For example, a series of strains have been constructed which contain useful selection markers in combination with a sterile mutation which greatly reduces their ability to mate with other yeast strains. Those strains (Table I) have been designated as SHY strains (11), and are suggested for use in applications where biological containment is advisable. In some cases it may also be desirable to make use of host strains which do not contain endogenous 2μm DNA plasmids [cir^o] and/or mitochondrial DNA [rho^o]. Several such strains now exist (Table II), and methods are available for the construction of additional ones as needed (12-18).

DNA VECTORS SUITABLE FOR MOLECULAR CLONING IN YEAST

Molecular cloning vectors constructed for use in yeast share in common the feature of containing at least one gene selectable in yeast. Beyond that single feature they vary widely in structure and concept of use. The vectors are of two basic classes: (a) those which contain no yeast replicator and transform yeast at low frequency (1-10 transformants per μg DNA) by integration into chromosomes at sites of sequence homology (8,9,19), and (b) those which contain an autonomously replicating sequence (*ARS*), transforming yeast at high frequency (500-20000 colonies per μg DNA) and can exist as free plasmids in yeast in one or many copies (19-22).

Examples of the first class (Figure 1) consist of an *Escherichia coli* plasmid (such as pBR322 or pMB9) into which has been cloned one or more fragments of yeast chromosomal DNA containing a gene useful for selection of successful transformants. Most of these vectors contain only single-copy yeast DNA and can be depended on to integrate at only that one location. However, some contain yeast repeated DNA such as ribosomal DNA (24), or 2μm DNA (23), in which case they transform with increased frequency and can integrate in multiple locations, still by homologous recombination.

TABLE I
SHY Yeast Host Strains (11)

Strain	Genotype
SHY1:	matα, ste-VC9, ura3-52, trp1-289, leu2-3, leu2-112, his3-Δ1, ade1-101.
SHY2:	matα, ste-VC9, ura3-52, trp1-289, leu2-3, leu2-112, his3-Δ1, ade1-100
SHY3:	mata, ste-VC9, ura3-52, trp1-289, leu2-3, leu2-112, his3-Δ1, ade1-101, can1-100
SHY4:	mata, ste-VC9, ura3-52, trp1-289, leu2-3, leu2-112, his3-Δ1

Plasmids of the second class (Figures 2 and 3) are more varied. Most of them also contain an E. coli vector and a gene or genes selectable in yeast, but in addition they contain a yeast autonomously replicating sequence ARS making them capable of existence as autonomous plasmids in yeast. The endogenous yeast 2μm DNA plasmid was used first as a replicator for recombinant plasmids in yeast since it was known to exist as an autonomous plasmid in its natural state (25-28). However, the 2μm plasmid contains no known selectable marker and is non-essential (29). It was therefore necessary to introduce into the plasmid other fragments of yeast DNA containing selectable genes, and these constructions were usually done in an E. coli vector to facilitate screening and DNA preparation. Included among the plasmids constructed were those which are illustrated in Figure 2. Comparison of the properties of the various plasmids revealed the positions of three functional regions on 2μM DNA: (i) the ARS; (ii) a function required for efficient replication of the ARS called REP; and (iii) a function required for intramolecular recombination within the inverted repeat called FLP. Plasmids in which REP is interrupted are replicated less efficiently in [cir^{o}] strains than plasmids in which it is intact, but can still transform at high frequency (23). Plasmids which contain a portion of 2μM DNA not including the ARS (CV3, Fig. 1) also transform [cir^{o}] hosts at comparably high frequency, presumably due to their homology to the multicopy plasmid permitting a high frequency of co-integrate formation, but fail to transform [cir^{o}] hosts except by chromosomal

TABLE II
Yeast Strains Lacking 2um DNA and/or Mitochondrial DNA

Strain	Genotype	Source/Reference
NNY1:	mat α, trp1, ura3-52, his3Δ-1, gal2, gal10/[cir°]	Stewart Scherer/(12)
DR19/T7-Ura3:	matα, his1, ura3/[cir°]	(13)
MC16L+1-39-8:	matα, ade2-1, his4-712, leu2, SUF2/[cir°]	(14)
LL20-11-2:	matα, leu2-3, leu2-112, his3-11, his3-15/[cir°]	(14)
6-1G-P188:	mata, his7, lys9/[cir°]	(15)
YF233:	matα, cyc, his3/[cir°]	(16)
YAT228:	mata, leu2, lys10, cyh^{r}, kar 1-1/[cir°]	(17)
YAT232:	mat α, leu2, lys10, cyh^{r}, kar 1-1/[cir°]	(17)
AH22:	mata, leu2-3, leu2-112, his4-519/[cir°]	(17)
NNY1-[rho°]:	mat α, trp1, ura3-52, his3Δ-1 gal2, gal10/[rho°]	Brad Hyman (Personal communication)

integration at very low frequency if at all. Plasmids in which FLP is interrupted fail to exhibit intramolecular recombination in [cir°] hosts. Plasmids containing functional FLP, or complemented in "trans" by a second plasmid containing FLP, recombine frequently within the 599 base pair inverted repeat, thereby generating a 1:1 mixture of two forms of the transforming plasmid, as was found to be true for endogenous 2μm DNA (16,23). The determination of these properties of 2μm vectors permit the choice or design of plasmids having features suitable for particular applications. For example, a 2μm vector which is ARS+ REP+ and FLP- introduced into a [cir°] host would be expected to be multi-copy (30-50 per cell), mitotically and meiotically stable, and would remain in a single sequence arrangement. Two examples of such plasmids are CV19 and CV20 (Figure 2) and new ones having different or additional selectable markers could be readily constructed.

Autonomous plasmids have also been constructed using ARS's derived from yeast chromosomal DNA (Figure 3). These plasmids are usually multi-copy in yeast, but are mitotically and meiotically

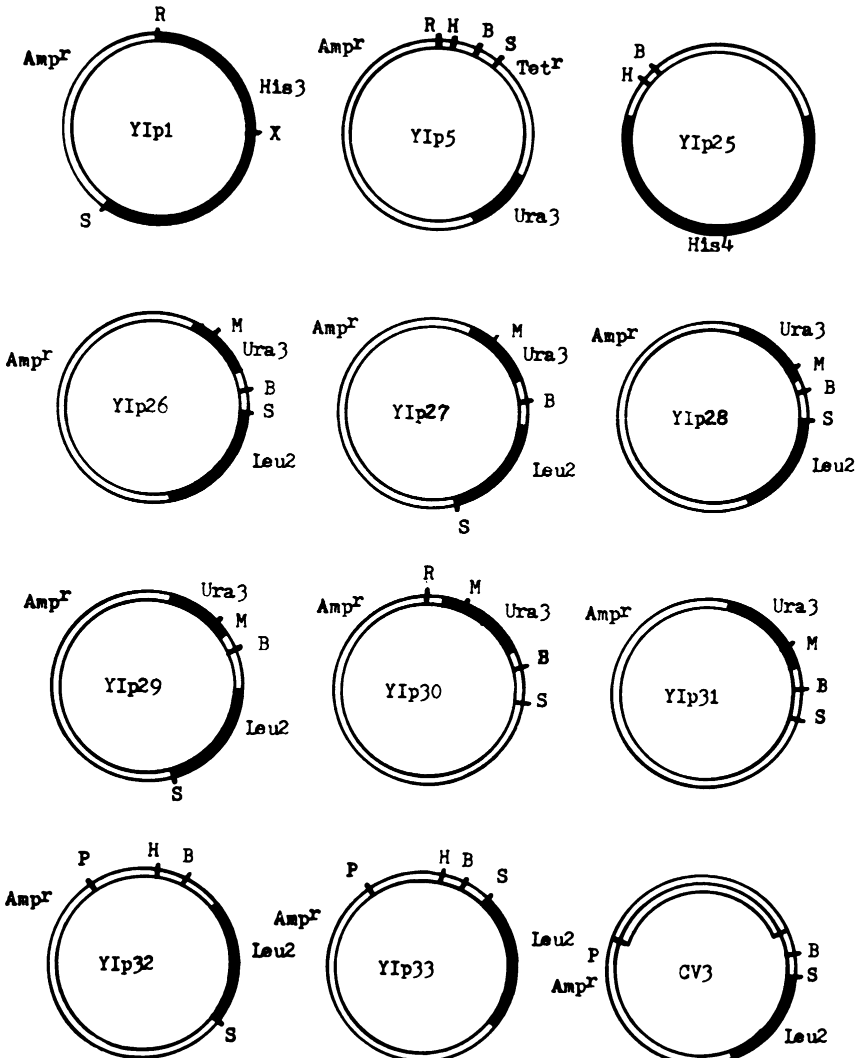

Figure 1. Type (a) Vectors Which Transform Yeast Only by Homologous Recombination With Endogenous Sequences. In the diagrams [open box] = pBR322 plasmid DNA, [solid box] = yeast chromosomal DNA, [double box] = yeast 2µm plasmid DNA, R= EcoRI, S=Sal1, X=XhoI, H=HindIII, B=BamHI, M= SmaI and P=PstI. References for the plasmids are as follows: YIp1 and YIp5 (19), YIp25 - YIp33 (11) and CV3 (23).

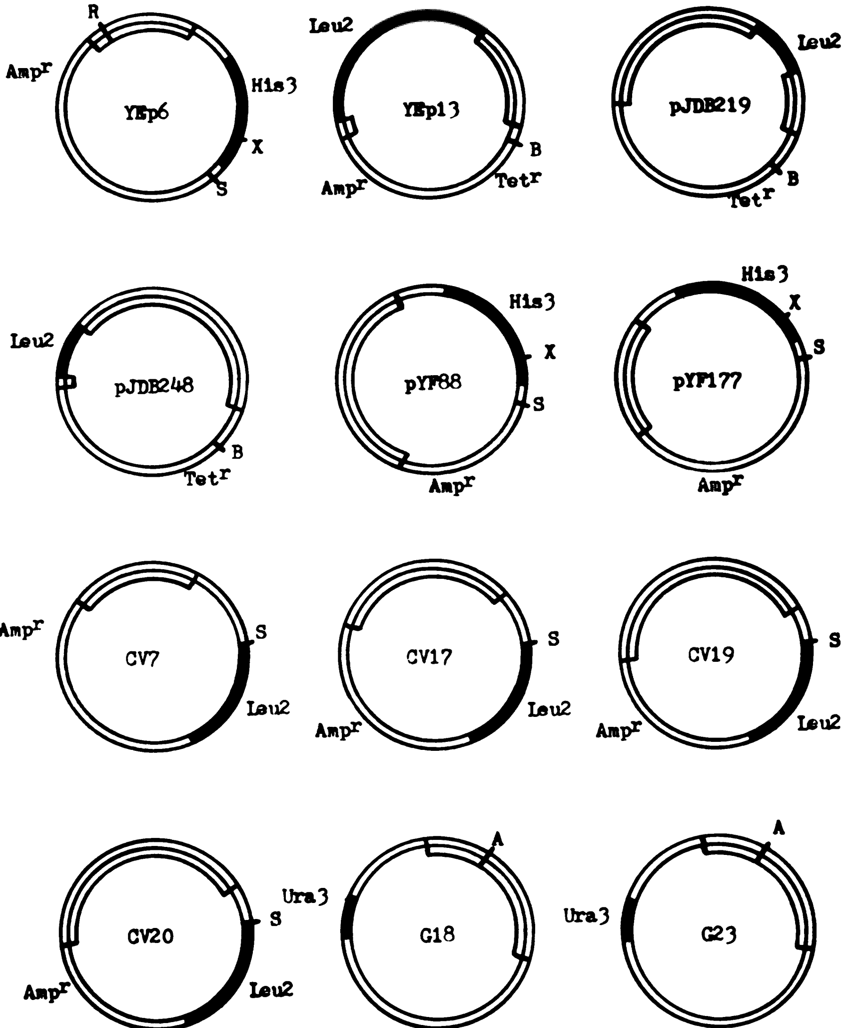

Figure 2. Type (b) Vectors Which Transform Yeast at High Frequency and are Maintained as Extrachromosomal Molecules Using the 2μm DNA Replicator. In the diagrams [open box] = *E. coli* plasmid DNA (pMB9 for pJDB219 and 248, pCRI for 618 and 23 and, pBR322 for all others), [solid box] = yeast chromosomal DNA, [double-line box] = yeast 2 μm plasmid DNA, R=EcoRI, X=XhoI, S=SalI, B=BamHI and A=HpaI. References for the plasmids are as follows: YEp6 (19); YEp13 (30), pJDB219 and pJDB248 (20), pYF88 and pYF177 (16), CV7, CV17, CV19 and CV20 (23), G18 and G23 (13).

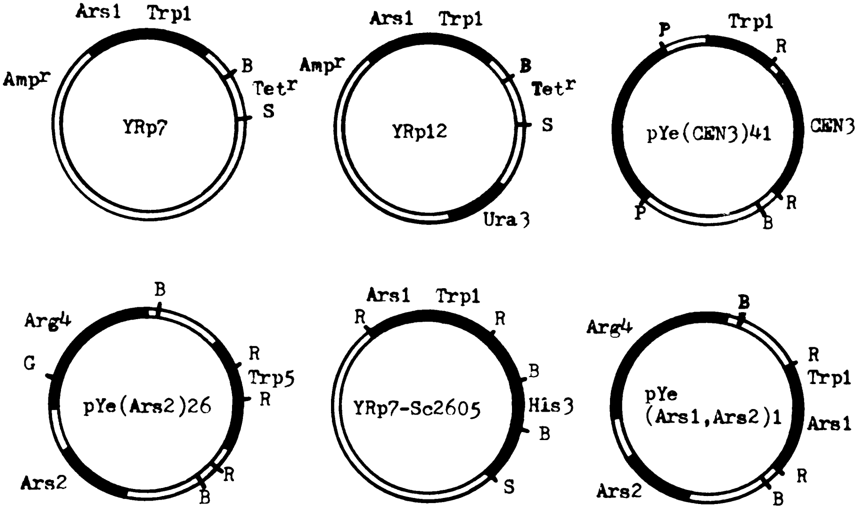

Figure 3. Type (b) Vectors Which Transform Yeast at High Frequency and are Maintained as Extrachromosomal Molecules Using Yeast Chromosomal Replicators. In the diagrams ▭ = pBR322 DNA, ▬ = yeast chromosomal DNA, B=BamHI, S=SalI, P=PstI, R=EcoRI and G=BglII. References for the plasmids are as follows: YRp7 (19); YRp12 (12); PYe(CEN3)41, pYe(ARS 2)26 and pYe(ARS,ARS)1 (31); YRp7-Sc2605 (32).

unstable, with the exception of those containing a yeast centromere such as pYe(CEN3)41 (31) (Figure 3). The centromere plasmids are relatively stable mitotically and meiotically, are probably present in one copy per haploid genome, and frequently segregate 2+ :2- in meiosis. There has been some confusion about the copy number of the chromosomal ARS plasmids not containing a centromere such as YRp7 and YRp12. They were first reported to be present in 1-10 copies per cell in a culture at stationary phase. However, we and others have observed that in stationary phase, only 5-10% of the cells in a culture grown under selection retain the plasmid. When the copy number of the plasmid per cell is adjusted for this low percentage, a copy number of more than 20-200 per cell is obtained. In addition, when cells containing YRp7 were harvested in mid-log phase, a time at which 40-60% of the viable cells contain the plasmids in a culture grown under selection for a plasmid carried gene, a mass of YRp7 plasmid DNA comparable to 2μm DNA was observed, indicating a copy number of 30-50 per cell in the population (data not shown). Therefore, the apparent low copy number of YRp7-like plasmids is due to instability rather than copy-number per se.

We have recently reported the construction of a plasmid, the TRP1 RI Circle (Figure 4), which contains a yeast chromosomal ARS and no centromere, but which is comparable in stability to the (CEN3)-containing plasmid (33, Zakian and Scott in preparation). The plasmid consists of the same 1.4 kilobase (kb) TRP1, ARS1,

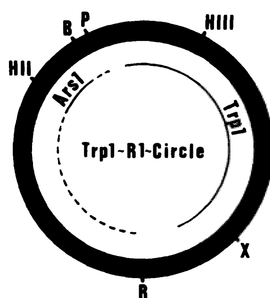

Figure 4. Trpl-Rl-Circle Partial Restriction Map. In the diagram ▬ = yeast chromosomal DNA, R=EcoRI, B=BglII, P=PstI, X=XbaI, HII=HindII, HIII=HindIII. The map is based on the complete sequence of the yeast Trpl EcoRI fragment determined by Tschumper and Carbon (34). Construction and properties of the plasmid are described elsewhere (33 and Zakian and Scott in preparation).

EcoRl restriction fragment of yeast chromosomal DNA as is present in YRp7. The fragment was excised from YRp7, circularized by intramolecular ligation, and returned to a trpl yeast strain by transformation and selection for Trp+. The TRPl RI Circle was found to be relatively stable mitotically and meiotically, was present in more than 100 copies per cell, and frequently segregated 4+ :0- in meiosis. In contrast to YRp7-like plasmids, the TRPl$^-$ RI Circle is retained in 75-85% of the cells in a stationary population grown with or without selection for Trp+.

Another plasmid having similar properties to TRPl RI Circle, and also consisting entirely of yeast DNA, but derived from 2µm plasmid, has been reported (35)(Figure 5). The 3.2 kb plasmid, pSLel, is a deletion derivative of pJDB219 (Figure 2), which contains only yeast sequences by virtue of deletion of all of pMB9 and much of 2µm DNA. This plasmid is also stable mitotically and meiotically and segregates 4+ : 0- in meiosis. However, the plasmid does not contain REP and is therefore expected to be less stable in [cir°] hosts.

Both TRPl RI Circle and pSLel have an additional advantage as vectors for genetic engineering in yeast. Since they consist only of yeast DNA, they are exempt from recombinant DNA restrictions, and remain so as long as only *S. cerevisiae* sequences are added to them.

ADDITIONAL STRATEGIES

In addition to the variety of vectors available having various stability, copy-number and replication properties, the potential exists for adding to those vectors sequences which promote expression and/or transport of the products of genes to be subsequently cloned in the vector. Expression enchancement could be achieved by adding inducible control sequences such as the alcohol dehydrogenase or galactose promoters. The gene to be expressed would then be inserted

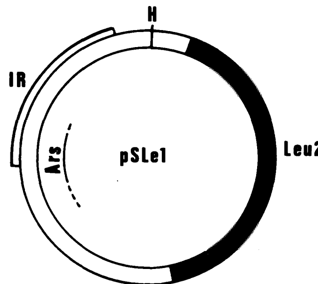

Figure 5. Partial Restriction Map of pSLe1. In the diagram ▭ = 2μm plasmid DNA, ▬ - yeast chromosomal DNA, IR indicates one of the 2μm DNA 599 base p air inverted repeats, H=HindIII. The map is based on the structure of pSLe1 described by Toh-E et al (35), the sequence of 2μm DNA determined by Hartley and Donelson (36) and the position of the 2μm DNA ARS determined by Broach and Hicks (23).

in the proper orientation next to the promoter so that its transcription could be enhanced by inducing the promoter. Export of the desired gene product might be accomplished by fusion of the gene in proper reading frame with the gene for a protein secreted from yeast such as invertase. The fused gene could then be inserted into an expression vector as in the preceding description, and the fused protein expressed might be exported by the mechanism used by the cell to secrete invertase.

DISCUSSION

The yeast host strains and vectors described in this paper are only a summary of those presently available. A number of additional ARS containing fragments functional in yeast have been isolated from yeast chromosomal DNA (37-39), as well as from DNAs of several other eukaryotes (38). Additional yeast centromeres have also been isolated, including (CEN4)(40). Colony banks of yeast chromosomal DNA fragments cloned in YRp7 are available (41). In principle, any yeast gene, for which a genetic selection is known, can be isolated as a cloned fragment by screening that bank. Therefore, since most of the vectors discussed have been constructed initially as *E. coli* recombinant plasmids, and all of the techniques of molecular cloning in that organism can be applied to the construction of additional ones, essentially any combination of genes, replicators and other known functional sequences can be constructed that one wants to spend the time and energy to construct.

This facility of construction and modification opens the possibility of designing special vectors to suit the needs of any particular molecular cloning application in yeast. Special host strains can also be constructed readily using a combination of molecular engineering and traditional genetics.

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DISCUSSION

Q. T. COOPER: In your genetic data you described segregation of the TRP circle with a pattern other than 4+:0-. In those cases can you distinguish segregation of TRP circle from loss of the plasmid during outgrowth of the ascospores?

A. JOHN F. SCOTT: No. Since the spores were germinated and permitted to form haploid colonies on non-selective media prior to testing the Trp phenotype, some of the Trp- clones may have resulted from segregation of the plasmid after germination.

Q. DONALD HELINSKI: In the case of several of the multi-copy *E. coli* plasmid cloning vectors the insertion of a segment of DNA lowers the copy number. Do you find this to be the case with your TRP1-R1 plasmid vehicle in yeast cells?

A. JOHN F. SCOTT: Yes. All fragments which we have cloned in the TRP1 R1 Circle thus far have resulted in reduced copy numbers and stability of the plasmid.

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TRANSFORMATION OF NEUROSPORA CRASSA UTILIZING RECOMBINANT PLASMID DNA

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INTRODUCTION

In comparison to yeast (15), the development of recombinant DNA technology for filamentous fungi is in its early stages. Recombinant DNA technology is dependent on two different though related matters: an efficient transformation system for the organism and an appropriate vector. In addition, transformation in any organism involves two processes: a system which permits the uptake of DNA into the cell and the subsequent integration of the DNA into a chromosome or its maintenance as a self-replicating entity in the recipient strain. An efficient transformation system for *Neurospora crassa* has been developed utilizing a cloned gene from Neurospora (4,27). The development of this procedure will be described here with the hope that some of the methods will be applicable to the development of such systems for other filamentous fungi.

Filamentous fungi are simple eukaryotic organisms which contain more DNA than either yeast or *E. coli*. The genome size of Neurospora is about two times larger than yeast (1.6×10^{10} daltons, 18, compared to 9×10^{9} daltons) and ca ten times larger than *E. coli* (2.6×10^{9} daltons, 24). Other filamentous fungi have amounts of DNA similar to Neurospora (e.g. *Schizophyllum commune*, 2.3×10^{10} daltons, 32). To be useful for genetic engineering an organism should have an already well-characterized genetic system with a variety of mutant genes that could be cloned into appropriate *E. coli* mutant strains. Neurospora has a wide variety of mutants located on seven well-mapped chromosomes. Standard genetic techniques are available, as well as tetrad analysis which permits accurate linkage determinations as well as the localization of centromeres. Unlike yeast and Aspergillus, there is no diploid

phase in the life cycle of Neurospora. However, dominance relationships between mutants can be determined by complementation in heterocaryons.

Transformation of Neurospora has been reported by various workers for a number of years (21,22,26) utilizing total Neurospora DNA as donor DNA. The recipient strains used in these transformation experiments involved an inositolless mutant (strain 89601, a cell wall mutant) combined with various morphological mutants. A more recent transformation procedure (34) has been developed based on the tendency of inositolless and osmotic mutants to make germ tubes resembling spheroplasts in media of high osmolarity. This procedure has not been readily reproducible and appears to be strain-specific since the ability of the recipient strain to be transformed readily can be lost by outcrossing. Both of these procedures depend on specific mutant types as recipient strains for the uptake of DNA. The transformation experiments to be described here do not require the presence of mutants affecting the cell wall.

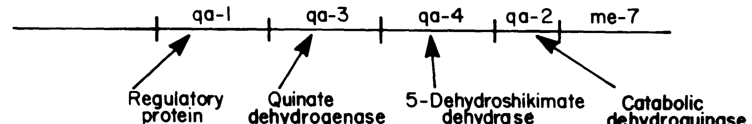

Figure 1. Gene order in the qa gene cluster in linkage group VII. The methionine-7 (me-7) gene is very closely linked to the qa-2 locus.

Qa Gene Cluster

The genetic system in Neurospora for which transformation was developed will be described briefly. We have been interested for a number of years in the genetic organization and regulation of the inducible quinic acid (qa) gene cluster in Neurospora (11). The catabolism of quinic acid is controlled by at least four genes which are clustered in linkage group VII very close to a methionine gene (me-7) and adjacent to the centromere (Fig. 1). Three of the four genes (qa-2, qa-3, and qa-4) encode inducible enzymes involved in the first three steps in the catabolism of quinic acid to protocatechuic acid in the utilization of quinic acid as a carbon source (Fig. 2). The fourth gene (qa-1) encodes a regulatory protein which acts in a positive manner to control the de novo synthesis of the other proteins of the qa gene cluster. None of the mutants in these qa genes can utilize quinic acid as a carbon source, but all grow well in the presence of sucrose. The inability of the qa mutants to grow on quinic acid and their ability to scavenge any other carbon source for growth has created problems in developing a direct transformation system for two of the mutants directly, qa-3 and qa-4. Mutants in the other two genes, qa-1 and qa-2, lack catabolic dehydroquinase, the enzyme involved in the conversion of dehydroquinic acid to dehydroshikimic acid (Fig. 2). This same reaction occurs in the aromatic biosynthetic pathway and involves one of the "genes" (arom-9) of the arom

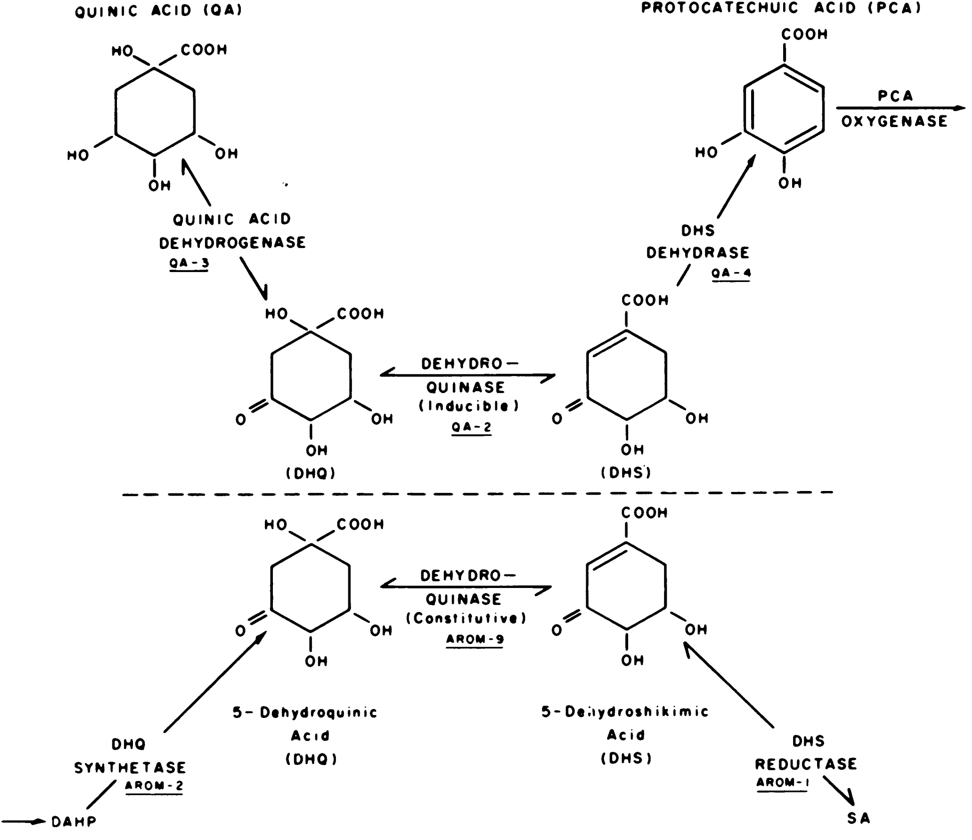

Figure 2. Relationships of the initial reactions in the inducible quinic acid catabolic pathway with the early reactions in the poly-aromatic biosynthetic pathway in Neurospora crassa.

"cluster gene" (10). Consequently, a double mutant with a block in the aromatic biosynthetic pathway (an arom-9 mutant) and with a block in either the qa-1 or qa-2 gene cannot grow on a minimal medium but requires an aromatic supplement for growth. This double mutant can be used like any other auxotroph as a recipient strain to select for transformation on a minimal medium.

The development of a transformation system for Neurospora was greatly facilitated by the cloning of the $qa\text{-}2^+$ gene in E. coli (33). This gene was cloned in E. coli by complementation of an $aroD^-$ recipient strain which lacks biosynthetic dehydroquinase. Subsequently, the entire qa gene cluster has been cloned in E. coli by selecting for $qa\text{-}2^+$ complementation of the E. coli strain utilizing the cosmid cloning procedure (27,28). Only the $qa\text{-}2^+$ gene has been found to be expressed in E. coli (28,33). By transformation utilizing appropriate recombinant plasmids and a variety of recipient strains of Neurospora, the genetic organization and regulation of the qa gene cluster is being examined at the molecular level.

TRANSFORMATION PROCEDURE

The transformation procedure for Neurospora has evolved over the past two years (4,28). This procedure, outlined in Table I, can be described in several steps, namely, germination of conidia and spheroplast formation, conditions for DNA uptake, and spheroplast fusion and regeneration of spheroplasts. The recipient strain contains a stable qa-2 mutation (M246), an arom-9 mutation and an insoitolless mutant (strain 89601). Although the inositolless mutant is present in the recipient strain, this procedure does not depend upon the inositolless mutant for transformation to occur.

TABLE I
Outline of Transformation Procedure

1. Spheroplasts are made by treating germinated conidia with glusulase in 1 M sorbitol.

2. DNA uptake occurs with spheroplasts in 0.4 ml 1 M sorbitol, 10mM MPOS pH 6.5, 50 mM $CaCl_2$, 5 μl DMSO, 50 μl PEG + 1-5 μg DNA pretreated with heparin in MOPS buffer on ice.

3. Fusion of spheroplasts in 40% PEG in MOPS.

4. Regeneration of spheroplasts in 1 M sorbitol, 3% agar, in a selective medium.

Germination of Conidia and Spheroplast Formation

A conidial suspension of no more than 5 x 10^6 conidia/ml in 150 ml of 1/2 concentration Fries minimal sucrose medium with appropriate supplements in germinated for 4 hrs at 25° in a 250 ml flask on a rotary shaker (28). The salt concentration of the minimal medium is reduced to half strength in order to reduce the calcium content. Preliminary experiments indicated that germinated conidia made spheroplasts more readily than ungerminated conidia. For the DNA to be taken up by the cell, the cell wall must be permeabilized. Glusulase has been used for a number of years to make protoplasts in Neurospora (1,31). The type of enzyme used to make spheroplasts will depend on the cell wall components. Various enzymes are currently used to make spheroplasts and protoplasts, e.g., glusulase and zymolyase for yeast, Trichoderma enzyme for Schizophyllum, and combinations of these enzymes (23). Spheroplasts are made in Neurospora by incubating the germinated conidia in 3% glusulase in 10 ml 1 M sorbitol for 70 min. at 30° on a slow shaker.

Conditions for DNA uptake

After incubation with glusulase, the spheroplasts are pelleted in a centrifuge and washed twice with 1 M sorbitol. The spheroplasts should be treated gently to prevent breakage. The third wash with 1 M sorbitol in MOPS buffer (4-morpholinepropane sulfonic acid, MOPS 10 mM pH 6.3, with 50 mM $CaCl_2$). Spheroplasts are then taken up in 0.4 ml 1 M sorbitol MOPS buffer, 5 μl DMSO (dimethylsulfoxide) and 50 μl PEG (polyethylene glycol, 4000 in MOPS buffer). DMSO has been used in the transformation of bacterial cells (19) as well as in chromosome-mediated gene transfer in mammalian tissue culture cells (20). While the spheroplasts are being washed, 1 to 5 μg of plasmid DNA is pretreated with 50 μl heparin (5 mg/0.5 ml in MOPS buffer) on ice. When the spheroplast suspension is ready, 0.25 ml of the suspension is then added to the pretreated DNA and incubated on ice for 30 minutes. Transformation will occur at room temperature (25°) but the level of transformation is 100 to 1000-fold less than when incubated on ice. Another essential factor for transformation is the ratio of spheroplasts to plasmid DNA. In the first experiments, 30 μg of plasmid DNA were used with 10^9 spheroplasts (4). This was too high a concentration of both spheroplasts and plasmid DNA for optimal recovery of transformants. Present experiments now utilize 1-5 μg plasmid DNA with 1 to 2 x 10^8 spheroplasts. Transformation will occur with a spheroplast suspension as low as 1 x 10^7 (Case, unpublished). In addition, not all spheroplasts are apparently compentent in taking up DNA.

Spheroplast Fusion and Regeneration of Spheroplasts

After 30 min on ice, 2.5 ml 40% PEG in MOPS buffer is added at room temperature. PEG is essential for transformation of Neurospora. If this step is omitted, no transformants are recovered. Also, no transformants are recovered if PEG is added and incubated on ice. After 20 min with PEG, the suspension is plated by adding aliquots to regeneration agar (1 M sorbiotl, sorbose-fructose-glucose Fries minimal medium and 3% agar) and overlayered onto normal 1.5% minimal agar plates. One M sorbitol must be present for regeneration of the cell walls. The agar concentration was found not to be as critical as the presence of sorbitol, but with 3% agar at least 50% more transformants were recovered than with 1.5% agar. Supplementation of the regeneration agar with low levels of yeast extract, casamino acids, and very low concentrations of aromatic amino acids, individually or in combination, did not enhance the recovery of *qa-2*$^+$ transformants.

Types of Transformants

Transformants can be separated into two different classes based on their ability to grow upon transfer to minimal agar slants: those that can continue to grow when transferred and those that

cannot grow when transferred, so called abortive transformants. The abortive transformants are interpreted as having some type of slowly-replicating plasmid-like entity that cannot replicate at an adequate rate and is ultimately diluted out during hyphal growth. With high transformation frequencies, 90% or more of the colonies found on minimal plates are of the abortive type. No abortive transformants were obtained from plasmid DNA which did not carry the wild type allele of the mutant gene in the recipient strain. In addition, abortive transformants are seen when total Neurospora DNA (no plasmid DNA) is used in transformation experiments (Case, unpublished). These results suggest that the presence of Neurospora DNA, and not the plasmid vehicle itself, is responsible for this class of transformants.

In the remaining tansformants which were able to grow upon transfer, the $qa\text{-}2^+$ gene was found to be integrated into the Neurospora genome. These transformants have been characterized by genetic analyses and Southern gel hybridization studies into three different types depending on the nature and the site of the integration event (4). The first type involves a simple replacement of the $qa\text{-}2^-$ gene with a $qa\text{-}2^+$ gene on linkage group VII. None of these transformants have pBR322 sequences integrated into the chromosome. In the second type of transformant, integration of the $qa\text{-}2^+$ gene occurs adjacent to the $qa\text{-}2^-$ gene. Only two transformants of this type has been characterized. One of these has pBR322 sequences integrated into the Neurospora chromosome. In both instances, the insertion of the $qa\text{-}2^+$ gene adjacent to the $qa\text{-}2^-$ gene has apparently resulted in inactivating the adjacent _qa-4_ gene. In the third class of transformants, the $qa\text{-}2^+$ gene has inserted at a site unlinked to the _qa-2_ region. About half of the transformants of this type has pBR322 sequences integrated at the same time. Of the 28 transformants analyzed genetically from pVK88, about half are replacement types, and half are unlinked insertion types (Case, unpublished). Tetrad analyses of transformants of the unlinked type containing pBR322 sequences indicate that the pBR322 sequences replicate and segregate in a normal Mendelian manner.

FACTORS AFFECTING NEUROSPORA TRANSFORMATION

Preparation of Plasmid DNA

In the purification of the plasmid DNA by the method of King et al (17), it is necessary to band the DNA three times on a cesium chloride ethidium bromide gradient. Although transformation in _E. coli_ will occur with relatively "dirty" DNA, transformation in Neurospora with "dirty" DNA will be 100 to 1000-fold less than with clean DNA. The contaminant is probably RNA which is competing with or interfering in some manner with the transformation process.

Preparation of Neurospora DNA

In preparing Neurospora DNA to be used in cloning experiments, the DNA should be isolated from wild type rather than from mutant strains unless there are special reasons for using DNA from mutants. In addition, the DNA should be isolated as rapidly as possible to prevent possible degradation by nucleases (4). The *qa-2*$^{+}$ gene has been cloned in *E. coli* using DNA from various mutant strains as well as from wild type. In utilizing these various recombinant plasmids for transformation in Neurospora, differences in the recovery of the *qa-4*$^{+}$ gene were observed. These results suggested that the differences might be related to strain differences or to the method of extraction of the DNA (28).

DNA Uptake by Spheroplasts

In the initial experiments, 30 min was chosen as an arbitrary time for incubation of the spheroplasts with plasmid DNA. To determine how rapidly DNA was taken up by the spheroplasts, a time course experiment from 5 to 40 min was performed (Table II). The results indicate that within 5 min DNA has been taken up by the spheroplasts on the basis of the recovery of transformants per µg DNA. In similar experiments, the time of incubation with PEG was examined. These results indicate that only 2 to 3 minutes incubation with PEG is necessary for transformation. Both the DNA uptake by spheroplasts and the effect of PEG are apparently very rapid.

TABLE II
Time Course Experiment - DNA Uptake by Spheroplasts Using Plasmid pES155.

Time in Min with DNA	*qa-2*$^{+}$ Transformants/ µg DNA*
5	110
10	140
20	160
30	155
40	140

*5 µg DNA used

Frequency of Transformation

In the first transformation procedure (4), the qa-2^{+} transformation frequency with plasmid pVK88 was 30 to 100 transformants/μg DNA. In subsequent experiments utilizing the procedure just described, 1000 to 5000 transformants μg DNA were obtained. The transformation frequency depends on the ratio of plasmid DNA to spheroplasts as well as on the density of spheroplasts when plated. The optimal recovery of transformants is dependent on a dilute concentration in the regeneration agar such that the non-transformed spheroplasts do not inhibit the growth of the transformed spheroplasts. This phenomenon has been previously observed for the recovery of revertants (12).

Attempts to Increase the Transformation Frequency

In yeast transformation experiments, cutting a circular plasmid into linear DNA fragments increased transformation frequencies (13). In Neurospora, pVK88 was cut with Xho which cuts once in the Neurospora DNA. The transformation frequncy in Neurospora was not increased using this linear DNA. In Neurospora, transformation experiments utilizing total Neurospora DNA cut with HindIII (linear DNA) and self-ligated after cutting (circular DNA), ten times more qa-2^{+} transformants were obtained with circular DNA than with linear DNA (Case, unpublished).

PLASMID VECTORS

Cosmid Technique

The cosmid technique developed by Collins and Hohn (7) provided a means of obtaining very large pieces of cloned Neurospora DNA by selecting for qa-2^{+} recombinant plasmids in the E. coli aroD^{-} strain. This procedure reduces the number of colonies necessary to obtain the whole Neurospora genome in a plasmid bank. With pBR322, the frequency of qa-2^{+} recombinant plasmids is one per 10^{5} transformants in E. coli, while one qa-2^{+} recombinant plasmid per 5000 transformants was obtained by the cosmid technique (7). The largest piece of qa-2^{+} Neurospora DNA cloned by this procedure is 36.5 kb and contains the entire qa gene cluster with flanking sequences (28).

Transformation Frequencies with Plasmids of Various Sizes

Since two different types of cloning vectors have been used, pBR322 and the cosmid vector, a variety of qa-2^{+} recombinant plasmids has been obtained which carry from 1 kb to 36.5 kb of Neurospora DNA. The transformation frequencies (transformants/μg DNA, Table III) indicate that the large plasmids apparently transform more efficiently than smaller plasmids. The limiting factor may involve the "competency" of the spheroplasts, since for any spheroplast there is a great excess of plasmid DNA during transformation.

TABLE III
qa-2^{+} Transformation Frequencies with Various Plasmids Increasing in Size with Respect to the N. crassa DNA insert

Plasmid	Vehicle	Insert Size of N. crassa DNA	Transformants/ ug DNA
pLHK11	pBR322*	1 kb	78
pVK57	pBR322	2.3 kb	42
pVK88	pBR322	6.8 kb	100
pMSK332	pHC79*	13 kb	726
pMSK331	pHC79	36.5 kb	512

*pBR322 = 4.3 kb; pHC79 = 6.43 kb

Ars, Autonomous Replicating Sequences

An ars sequence from Neurospora has been introduced into yeast (30). The ura plasmid of yeast does not normally replicate autonomously. By shotgunning total Neurospora DNA into this yeast plasmid, a chimeric plasmid was obtained which permitted the ura plasmid to replicate autonomously in yeast. When the qa-2^{+} gene from Neurospora was inserted into this chimeric plasmid containing both the ura^{+} gene from yeast and the ars sequence from Neurospora, the resulting plasmid was unable to replicate autonomously in Neurospora on the basis of the behavior of qa-2^{+} transformants selected in Neurospora (Huiet, Selker, and Case unpublished).

Self-replicating Neurospora Plasmids

There has been no report as yet of any self-replicating Neurospora plasmids comparable to the 2 μ circles found in yeast (3). There has been a report of a plasmid found within the mitochondria of a wild type strain of Neurospora isolated from nature (8). It is not yet known whether this plasmid would be usable as a vector for transformation in Neurospora. In addition, there has been a report of a plasmid found in mitochondria of Podospora anserina which a involved in the senescence of the organism (29).

Cloning of a Neurospora Centromere

The qa gene cluster is located very close to the centromere in linkage group VII. It is possible that one of the plasmids [isolated by the cosmid procedure which contains the 36.5 kb fragment of Neurospora DNA] may actually include the centromere. Northern gel analayses indicate that a number of other messages are present on these fragments in addition to the messages involving the qa gene cluster (V. Patel, unpublished). As indicated earlier, involving the qa gene cluster (V. Patel, unpublished). As indicated earlier, there is no evidence that any of these plasmids are stably self-replicating. Only when the genes on the other side of the centromere have been cloned on a contiguous piece can we be certain that the centromere of linkage group VII has been cloned. It would be useful if a cloned centromere from Neurospora could be obtained together with an ars sequence, since this structure should behave as a self-replicating mini-chromosome just as in yeast (5). It would be interesting to take one of the large plasmids which may contain the Neurospora centromere and clone this DNA into yeast to determine if the Neurospora DNA in yeast would act like a centromere and be self-replicating in yeast. One of the problems in cloning a centromere in Neurospora is whether Neurospora will tolerate any type of prolonged aneuploidy, even involving a small amount of extra DNA. Pseudo-wild types (disomics) occur quite readily in Neurospora, but they are unstable and breakdown very quickly to the stable haploid chromosome number. Barry (2) has cytological evidence for the replication of an acentric fragment in Neurospora through a number of cell divisions. How long such a fragment could be maintained has not been determined.

Cloning of Other Genes from Neurospora

The only published information concerning cloned genes from *Neurospora crassa* involves the ribosomal genes (9) and the qa gene cluster. David E. A. Catcheside (per. comm.) has cloned the $qa\text{-}2^+$ gene independently using λ phage. Several experiments have been performed to clone other genes from Neurospora by complementation with appropriate *E. coli* strains, but so far these results have been negative (Kushner, unpublished). The high frequency of transformation now obtainable in Neurospora should make it possible to generate recombinant plasmids and to select directly by transformation in Neurospora for expression of Neurospora genes which might not be able to complement *E. coli* mutants. This procedure has worked effectively for the $his\text{-}4^+$ gene of yeast (14).

Intergeneric Transformation for Gene Expression

If a particular gene has not been cloned in *E. coli*, then intergeneric transformation with recombinant plasmids carrying genes from yeast (e.g. $leu\text{-}2^+$, $his\text{-}3^+$, $ura\text{-}3^+$, or $trp\text{-}1^+$, 25)

might be used to transform comparable mutants in Neurospora. However, chimeric plasmids containing the qa-2$^+$ gene from Neurospora combined with each of four genes (trp-1$^+$, ura-3$^+$, leu-2$^+$, or his-3$^+$) from yeast have not been effective in transformation of the comparable mutants in Neurospora (L. Huiet, unpublished). In these experiments, qa-2$^+$ transformants were obtained, but the yeast gene was not expressed in any of the transformants. Although to date intergeneric transformation has not been effective for Neurospora, it might work for other fungal species. In performing such experiments, the yeast plasmid should contain some DNA from the organism to be transformed in order that there be some homology between the donor and recipient DNAs.

Expression of Bacterial Genes in Neurospora

It would be useful to have an *E. coli* plasmid gene expressed in Neurospora since this would provide a selection system permitting the easy detection of the plasmid. Both the chloramphenicol resistant gene and resistance to G418 are expressed in yeast (6,16). A plasmid has been constructed carrying both of these genes as well as the qa-2$^+$ gene Neurospora (Vapnek, unpublished). Neurospora reverts at a high frequency spontaneously for both chloramphenicol and G418 resistance. Using a chloramphenicol sensitive qa-2$^-$ recipient strain and selecting for qa-2$^+$ transformants, no qa-2$^+$ transformants of over 100 tested were able to grow readily in the presence of both antibiotics. Southern gel analysis is being done to determine if only the qa-2$^+$ gene has been integrated, or if the entire plasmid has been integrated and the plasmid genes are not expressed in Neurospora.

SUMMARY

This discussion describes the present status of transformation and genetic engineering in Neurospora. It also indicates some of the problems which have been encountered and what approaches were made to solve these problems. The use of recombinant DNA techniques has permitted the cloning of the entire qa gene cluster, and has provided much additional evidence at the molecular level concerning gene structure and regulation of this gene cluster in Neurospora.

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DISCUSSION

Q. Do the genes in the Qa cluster contain intervening sequences?

A. CASE: The *qa2* gene does not contain any intervening sequences since it is expressed in *E. coli*. Since the other two genes are not expressed in *E. coli* by complementation with appropriate *E. coli* strains, we are presently in the process of trying to determine if these genes contain intervening sequences.

Q. Are the genes from yeast and *E. coli* which have been integrated into the Neurospora genome transcribed?

A: CASE: We do not know whether the yeast and the *E. coli* genes are transcribed in Neurospora. The appropriate *Northern* gel by oxidization studies have not as yet been done. By *Northern* gel analysis, my graduate student Layne Huiet does know that the *qa2*$^+$ gene is not transcribed in yeast. Yeast transformants which contain the *qa2*$^+$ gene have been characterized for qa2$^+$ gene expression from a *trp-1*$^+$ *qa2*$^+$ climeric plasmid selected for by transformation of a *trp-1*$^-$ strain in yeast.

PERSPECTIVES FOR THE GENETIC ENGINEERING OF HYDROCARBON OXIDIZING BACTERIA*

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Hydrocarbons have many different structures, but these microbial substrates have two general characteristics which influence the nature of oxidation pathways. (1) Hydrocarbons are usually hydrophobic molecules, and oxidizing activities are consequently located in cellular membranes. (2) Hydrocarbons are composed of several basic structural units (such as aromatic rings and aliphatic chains). In order to break down these composite substrates, oxidation pathways are organized into segments that are conveniently labeled "throats" and "stomachs". Throats contain the initial activities which convert a specific hydrocarbon substrate into one of a few common intermediates, such as fatty acids or catechols. Stomachs contain the subsequent activities which transform the intermediates into the organic acid substrates of central metabolic pathways. Typical stomachs include β-oxidation and catechol ring fission pathways. Detailed genetic analysis has been carried out for a few hydrocarbon oxidation pathways, including the plasmid-borne _alk_ and _xyl_ systems for oxidation of n-alkanes and of toluene or xylenes. In both cases, there is duplication of plasmic-and chromosome-encoded activities at certain steps of the oxidation pathway. Induction specificities in both systems are often more important than enzyme specificities in determining growth phenotypes on different substrates. Oxidation of halogenated aromatic substrates, such as chlorobenzoic acid or the pesticide 24D, has been

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the subject of less detailed genetic study, but haloaromatic pathways illustrate the same basic principles as those for non-halogenated substrates: (i) the need to match throats and stomachs, and (ii) the importance of proper pathway regulation to ensure substrate catabolism. The use of broad host-range cloning vectors means that recombinant DNA methods are available for use with hydrocarbon-oxidizing gram-negative bacteria. However, detailed consideration of the possible rate-limiting steps in hydrocarbon oxidation indicates that cloning and overproduction of particular enzymes may often not be successful in strain improvement for specific biodegradations or bioconversions. A particular problem can be the toxicity of membrane hydrocarbon-oxidizing proteins when they are produced at very high levels.

DISCUSSION

Q. JOHNSON: Is there evidence of an association of plasmids with the membrane?

A. SHAPIRO: Many plasmids are associated with membrane during replication. It is not yet known whether there is any specific association of plasmid hydrocarbon oxidation determinants with the cytoplasmic membrane.

Q. KUNZ: You mentioned the highly coordinated manner in which pathways ("throats and stomachs") for aromatic compounds are regulated. Is there any indication that pathways for aliphatic compounds which proceed via fatty acids are also highly coordinated in contrast to the case that you cited for alkane degradation, wherein enzymes for fatty acid metabolism (β-oxidation) are constitutively controlled?

A. SHAPRIRO: The only system I know of is the β-oxidation pathway of *P. putida*, which is expressed constitutively. That does not mean, however, that it is not regulated.

PHOTOSYNTHESIS AND CLONING IN CYANOBACTERIA

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INTRODUCTION

Research into photosynthesis is now a century old. Progress in the understanding of this mechanism has been slow, primarily for two reasons. First, the process is highly complex and involves dozens of components which must be arranged in a precise, orderly and structured fashion. Secondly, the so-called light-reactions of photosynthesis with which we will be concerned are located within a membrane. Unfortunately, techniques for the study of membrane-localized structures have lagged behind other fields and have only come into their own during the past 10 years. Another feature that has tended to impede progress in photosynthetic research concerns the wide range of organisms that can be utilized for such studies. Thus, researchers have worked with plants, eukaryotic algae, photosynthetic bacteria, and the cyanobacteria (blue-green algae); specific organisms were seen as possessing certain important characteristics and were used only during specific experiments. Though the cyanobacteria have often been utilized as an organism in photosynthetic research, the range of experiments have been somewhat narrow. These include an assessment of the phycobiliproteins in light-harvesting as well as comparative biochemical studies.

The most significant advance in the understanding of the photosynthetic process was the work that culminated in the promulgation of the Z-scheme. This now generally accepted scheme indicates that light energy is transduced into chemical energy through the involvement of two photochemical pigment systems which operate in series and which are connected by the electron transport chain. This now provides a framework which can be used to interpret many results. Nonetheless, the Z-scheme is still much like a house in the early

stages of construction--the foundation and the outlines of the structure are visible, but much of the details remain unknown. A quick glance at even the most recent versions of this scheme will indicate that most of the proposed components are known only by their redox potential or their characteristic absorbance features. Much remains to be done in specifically identifying the components, learning how they are arranged in the membrane and ultimately correlating this information with photosynthetic function.

One very important approach to the study of such problems is, of course, genetics. Over the years, photosynthetic mutants have been isolated in plants and algae and used to probe many aspects of photosynthetic electron transport. But, such mutants have been difficult to isolate and often even more difficult to understand. The work has been productive, but limited by many technical and biological problems. In particular, it has not been readily possible to manipulate genes between cells and carefully study the control of membrane assembly. The purpose of this paper is to describe our efforts during the past few years to develop a genetic system in *Anacystis nidulans* that would be useful in attacking numerous probelms in photosynthesis. This, in turn, is the basis of a long-term strategy to clone those genes coding for photosynthetic membrane proteins.

The Cyanobacteria

The cyanobacteria are ancient organisms that have only recently come to the attention of many molecular biologists. These organisms are photosynthetic prokaryotes which contain a photosynthetic apparatus remarkably similar in all respects to that of a eukaryotic chloroplast. The cyanobacteria are readily distinguishable from the two other major groups of photosynthetic bacteria, the purple and green bacteria, by the nature of their photosynthetic pigment system and by their performance of oxygenic photosynthesis. Thus, the cyanobacteria possess some rather unique attributes for the study of photosynthesis.

Why then have the cyanobacteria been utilized in a rather narrow range of experiments that have ignored some of their principal benefits? One reason for this is a reflection of the historical view of these organisms as algae. The possession of the range of pigments characteristically associated with algae gave emphasis to their essentially plant-like mode of photosynthesis and led most botanists to set them completely apart from the photosynthetic bacteria. During the last 20 years, a completely different view of the phylogenetic relationships among photosynthetic micro-organisms has become established. The blue-green algae and bacteria are seen as a coherent group, the prokaryotes, which characteristically lack-membrane-bound organelles such as nuclei and mitochondria and which

do not have their genetic material located on more than one chromosome in a membrane-bound nucleus (13,35,37). This relationship has been confirmed on the basis of their nucleic acid biochemistry, fine structure, and cell wall chemistry (11,12,35,36). Once these organisms are considered as bacteria, it becomes obvious that they are amenable to the same broad range of experimentation. This is the starting point for the strategy to be developed below.

The fact that the cyanobacteria are the only prokaryotes capable of aerobic photosynthesis gives rise to important experimental properties that are advantageous to photosynthetic research. They contain only one type of internal membrane, the photosynthetic lamellae, which is easy to isolate. They are easy to grow and manipulate biochemically, and the unicellular organism discussed here can be cloned as single colonies on agar plates. In addition, it is likely that all of the genetic information for membrane structure and function is encoded by a single genome. This greatly simplifies studies on the control and regulation of genetic function. Thus, it would seem that the cyanobacteria should be an ideal genetic system for photosynthesis. This has not been true until recently for two interrelated reasons. First, many strains are essentially obligate photoautotrophs, and photosynthetic mutants would tend to be lethal. Secondly, no reliable means for the transfer of genetic information has been available. One of our most important objectives over the past 2 years has been to overcome these difficulties, and this goal has now been accomplished. We report here on the progress made in a number of laboratories that has led to the development of a coherent genetic system in one cyanobacterial strain, Anacystis nidulans. We show that this progress leads directly to a system for the cloning of photosynthetic genes.

Photosynthesis and Cyanobacterial Physiology

Although the photosynthetic mechanism forms the central focus of our work, space limitations allow only a cursory description of this process (Figure 1). The presently accepted scheme for the light reactions in "green-plant" photosynthesis is a chlorophyll-mediated, light-driven mechanism which ultimately results in the production of ATP, the reduction of $NADP^+$, and the evolution of oxygen. In considering this process, it is convenient to divide the mechanism into three segments: (1) the light-harvesting apparatus and the photochemical pigments; (2) the electron transport chain; and (3) phosphorylation. The light-harvesting apparatus in cyanobacteria consists of pigments such as chlorophyll a and the accessory pigments called phycobiliproteins. Their function is to funnel incoming photons to the specialized chlorophylls that are involved in the photochemical reactions. Aggregates composed of the light-harvesting pigments and the reaction centers are called photosystems (PS). There are two functionally and structurally

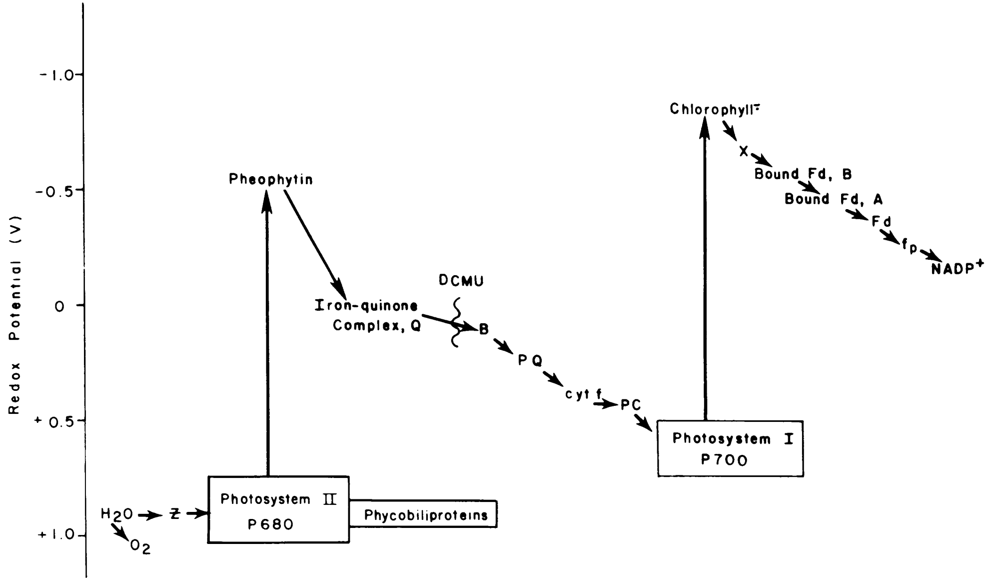

Figure 1. Energy diagram (Z-scheme) of photosynthetic electron transport plotted in terms of the oxidation-reduction potentials of the interacting redox components. The scheme is drawn to emphasize that 2 light reactions, acting in series and connected by an electron-transport chain, are needed to move an electron from H_2O to $NADP^+$. This diagram is not all-inclusive and omits some components whose functional role is still controversial. The components include: B, a secondary acceptor of Photosystem II; PQ, plastoquinone; cytf, cytochrome f; PC, plastocyanin; chlorophyll$^{\cdot-}$, a chlorophyll anion radical that is proposed as an intermediate electron acceptor of Photosystem I; Fd, ferredoxin; fp, ferredoxin-$NADP^+$ reductase. P680 and P700 are the reaction center chlorophylls of Photosystem II and I, respectively.

distinct photosystems in aerobic photosynthesis which absorb light of somewhat different wavelengths; in particular, far red light (light with wavelengths above 680nm) can only be absorbed by PSI. These photosystems act in series and are connected by the electron transport chain. Electrons excited during photochemistry can be transported spontaneously down the electron transport chain. Protons are also transported across the membrane leading to a strong trans-membrane pH gradient. This high-energy state provides the energy for ATP synthesis.

Light absorbed by PSII excites an electron that can ultimately pass down the electron transport chain twoards PSI, a process which is strongly inhibited by the herbicide DCMU. The chlorophyll in

PSII is ultimately restored to its original state by an electron originating from H_2O. This water-splitting step results in the evolution of O_2. Long wavelength light is absorbed by PSI and this excitation results in the reduction of $NADP^+$ to $NADPH_2$. These processes can be coupled to phosphorylation in one of two ways. In non-cyclic photophosphorylation, the formation of ATP depends on the functioning of the entire scheme depicted in Figure 1. In cyclic phosphorylation, ATP formation is coupled with an electron shunt about PSI; no O_2 is evolved, nor is $NADP^+$ reduced. This process is insensitive to DCMU and only requires far red light (14,18).

The electron transport chain is composed of carriers such as cytochromes, plastoquinone, ferredoxins, ferredoxin-NADP reductase, and, presumably, a number of other yet-unidentified components. Although some of these compounds have been isolated and characterized, there is little doubt that our present knowledge of the structure of the photosynthetic membrane is meager indeed. We have detected well over one hundred polypeptides in the cyanobacterial photosynthetic membrane (27); however, the functional identity of less than two dozen is known with certainty. The primary acceptors of the individual photosystems is a case in point. Until recently, they were referred to merely as Q (PSII) and X (PSI). Biophysical measurements now indicate that Q may be an iron-quinone complex (24) and that the primary acceptor in PSI may be a chlorophyll anion which, in turn, reduces bound ferredoxin centers (28). Despite these important findings, the physical nature of the polypeptides associated with these components is unknown. The genetic approach to be discussed below should be most valuable in the identification of these physical structures.

Members of the cyanobacteria are aerobic photoautotrophs in which the photosynthetic reactions are used to drive biosynthesis at the expense of inorganic nutrients. However, many strains can grow photoheterotrophically or chemoheterotrophically on other sugars metabolized via the oxidative pentose phosphate cycle (11,35). Such heterotrophic growth in the dark is always much slower than photoautotrophic growth but it can be significant in determining overall strategy. Heterotrophic capability can be an important criterion. Many strains capable of reasonable heterotrophic growth are filamentous and are less favorable for genetic studies. However, within the unicellular strains, Aphanocapsa 6714 shows good facultive photoautotrophic and heterotrophic growth (34). Therefore, it would seem that Aphanocapsa would be the organism of choice for genetic studies of photosynthesis; the isolation of non-conditional lethal, photosynthetic mutants should be possible. In addition, transformation has been demonstrated in Aphanocapsa 6714.

Unfortunately, the potential of this organism has been mitigated by some technical problems. First, the transformation system is

relatively inefficient and is totally dependent on the precise growth phase of the cells (1). Furthermore, Aphanocapsa is very difficult to mutagenize (2) and is rather poorly sensitive to lysozyme. More importantly, attempts to enrich for photosynthetic mutants with the techniques to be discussed later have not been possible, since the compounds kill Aphanocapsa equally well in the light and in the dark (Astier and Joset-Espardellier, personal communication). Nonetheless, the potential benefits of heterotrophy are important and we are presently searching for procedures that can generate photosynthetic mutants in Aphanocapsa.

In the absence of an ideal heterotrophic strain, we have chosen to work with the autotrophic strain Anacystis nidulans R2. Therefore, we have had to isolate temperature-sensitive photosynthetic mutants in addition to herbicide-resistant strains. Though this system has limitations, we feel that it represents the best compromise at the present time. A. nidulans R2 grows well in liquid culture, it plates well on agar, it has a lysozyme-sensitive cell wall, and exhibits a high frequency of transformation. In the following sections, we will detail the strategy that will ultimately be used in A. nidulans for the cloning of genes coding for photosynthetic functions. We will focus on three subjects:(1) the procedures used for the isolation of temperature-sensitive, photosynthetic mutants; (2) transformation in selected unicellular cyanobacteria; and (3) plasmids and the construction of hybrid cloning vectors capable of replication in Escherichia coli and Anacystis nidulans. We will conclude with a discussion on our strategy for the cloning of specific proteins involved in photosynthesis.

RESULTS

Selection and Enrichment of Mutants

Photosynthetic mutants have been isolated in a number of green organisms, particularly in the green algae, Chlamydomonas reinhardtii (20) and Scenedesmus obliquus (6,21). More recently, mutants of maize and barley (22) have been analyzed in some detail. The major hurdle in all of these studies has been techniques to select and enrich for photosynthetic lesions. One of the most successful selection procedures has been the fluorescence procedure devised by Bennoun and Levine (4) for C. reinhardtii and used by Miles (22) in maize. This technique relies on the observation that some colonies or plants defective in photosynthesis emit an abnormally high (or low) level of chlorophyll fluorescence. The fluorescence of a large population of colonies can be screened quickly during illumination with ultra-violet light. If a cell has a lesion on the reducing side of PSII, an enhanced level of fluorescence will result; conversely, lesions on the oxidizing

side will yield a decreased fluorescence.

We have generated temperature-sensitive, high fluorescence mutants in the cyanobacterium, Synechococcus cedrorum, using this procedure (30,31). Most of these mutants are photosynthetic and we will describe the properties of one such mutant in the following section. However, the procedure is tedious and inefficient and many colonies must be screened.

Based on this experience, we decided that the best approach would be to obtain a procedure to selectively enrich for mutants defective in the electron transport mechanism. We began a search for redox-active compounds that would interact with the electron transport chain (5). We hoped to find a compound that was cytotoxic to photosynthetically active cells, but that had no effect on photosynthetically-defective cells. As shown in Figure 1, the electron carriers have redox potentials that range from +0.8V for PSII to -0.8V for the primary acceptor of PSI (28). Theoretically, any compound that had the appropriate redox potential could accept electrons from a specific carrier. For example, if we had a compound with a potential of -0.6V, it would be highly likely that it would interact with a component on the reducing side of PSI. In this case any defect in the chain from H_2O to this carrier would fail to reduce the compound and would protect the cell. After unsuccessful attempts with methyl viologen and diquat, two chemicals known to interact in this region, we tried the drug metronidazole.

Metronidazole (2-methyl-5-nitroimidazole-1-ethanol) is a redox active nitroheterocyclic antibiotic (E_o' = -525mV at pH 6.9) that is used in the treatment of anaerobic bacterial infections (3, 26). It was first utilized in photosynthetic organisms by Schmidt et al. (29) to enrich for electron transport mutants in C. reinhardtii. We determined that metronidazole acted as an effective electron acceptor in both whole cells and membranes and interacted with the electron transport chain at the level of ferredoxin (15). The key question was whether or not the compound could discriminate between photosynthetically and non-photosynthetically growing cells. To measure this discriminatory power, we analyzed the ability of cells treated in the light or in the dark to survive metronidazole treatment.

The most critical results concerning metronidazole action are shown in Figure 2 and Table I. Incubation of cells for 6 hours at 40°C in 1mM metronidazole causes a dramatic reduction in cell viability. The toxic effect in the light is fully 5 orders of magnitude greater than that seen during an incubation in the dark. The dark treatment generally resulted in a killing of 80%; however, this could be decreased significantly be incubating cells in the dark for 24 hours prior to metronidazole treatment. Table I demonstrates that photosynthetic electron transport is required

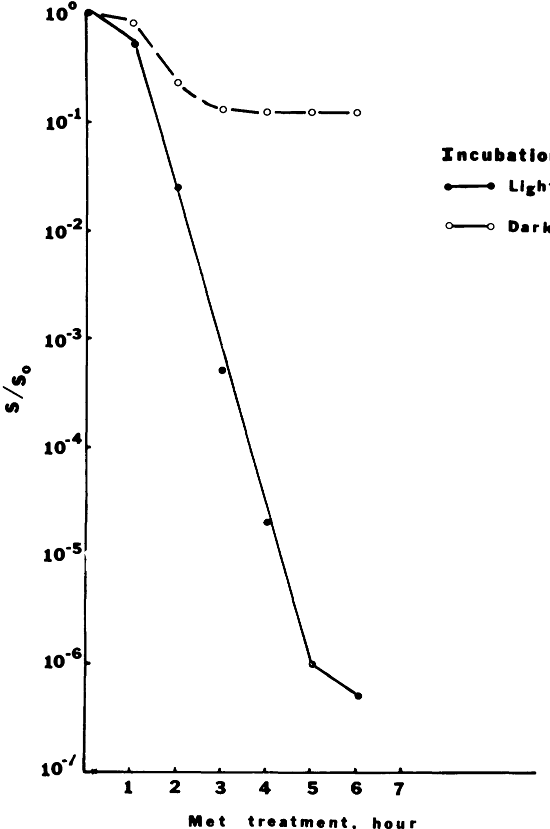

Figure 2. Effect of metronidazole on cell viability of S. cedrorum cells. Cells (5 x 10^8 cells/ml) were incubated at 40°C with 1mM metronidazole. Aliquots were centrifuged to remove the drug and were plated. S/S_o is the ratio of colonies recovered during the treatment to those recovered prior to incubation (15).

to obtain maximal killing. A cessation of photosynthetic electron flow induced by darkness, by DCMU, or by a mutation blocking electron transport decreased the killing by five orders of magnitude. Thus, metronidazole discriminates very effectively between photosynthetic and non-photosynthetic cells and should be an excellent enrichment agent for the isolation of photosynthetic mutants.

Our findings indicated that the reduction of metronidazole and concomitant toxicity required electron transport from H_2O to the level of ferredoxin, implying that defects throughout that portion of the chain should be detectable. The rationale for the use of metronidazole in the isolation of temperature-sensitive, photosynthetic mutants is as follows. During photosynthetic electron

TABLE I
Effect of Photosynthesis on Metronidazole Killing

Strain	Incubation	% survival
wild-type	Light	6.7×10^{-5}
wild-type	Dark	16
wild-type	Light + 10^{-5}M DCMU	12
wild-type	Dark + 10^{-5}M DCMU	15
ts-53 (temperature-(sensitive mutant)	Light	19
ts-53	Dark	22

Incubation was in BG-11 at 40°C for 6 hours.
From Guikema and Sherman (15).

transport, metronidazole is reduced with the concomitant formation of toxic products. When a cell population is mutagenized and then raised to the non-permissive temperature, mutants defective in electron transport will be unaffected by metronidazole. In this way, most of the competent cells will be destroyed, enriching the survivors for photosynthetic mutants.

The protocol for the isolation of temperature-sensitive mutants using metronidazole enrichment is summarized in Figure 3. For comparison, the high fluorescence selection procedure is also listed in this figure. The rationale for these steps is established (15,30,31). It is interesting to compare the relative efficiencies of the two techniques. In the metronidazole enrichment procedure, a total of 6000 surviving colonies were replica plated in a series of experiments. Sixty colonies were temperature-sensitive, and, of these, 31 separate strains had abnormalities in photosynthetic properties. These figures represent large increases over our previous attempts at the isolation of temperature-sensitive photosynthetic mutants. Using the high fluorescence selection procedure, we initially obtained 8 mutants out of 20,000 colonies screened. The finding that at least 1% of the population consists of temperature-sensitive mutants and that 50% of the total number of these mutations are photosynthetic shows the effectiveness of the metronidazole enrichment procedure.

There were two aspects of our results that we found puzzling. First, we thought that we should generate a higher proportion of mutations within the surviving population. Secondly, a relatively high percentage of the photosynthetic mutants were localized in and around PSI (15). We solved both problems by studying the turnover rate of the proteins involved in specific photosynthetic functions.

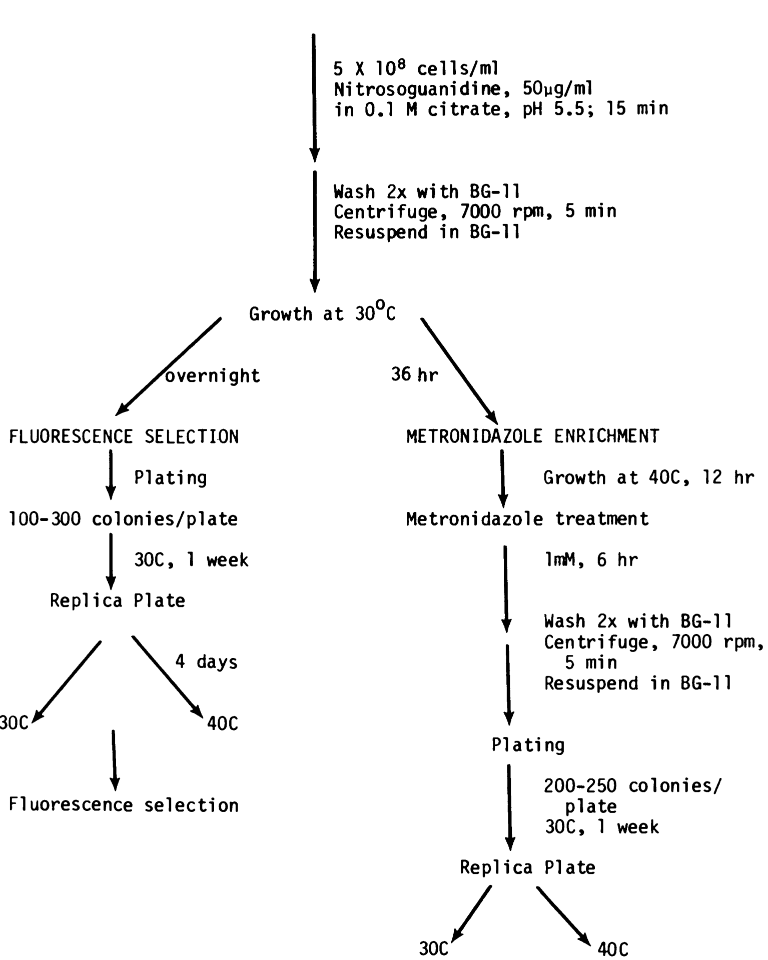

Figure 3. Protocol for the isolation of temperature-sensitive mutants in S. cedrorum and A. nidulans using the high-fluorescence procedure (left) and metronidazole enrichment (right).

We discovered that PSII proteins turn over more slowly than most PSI-associated proteins. Therefore, we modified the metronidazole procedure by growing the cells for 36 hours at 30°C before shifting the culture to 40°C. Using this modified protocol in a mutagenesis experiment with the transformable strain Anacystis nidulans R2, we obtained over 40 temperature-sensitive mutants from a total of about 600 surviving colonies. Again, over half of these show photosynthetic abnormalities; importantly, many more of these mutants are PSII defective. We now possess a protocol that generates numerous mutations through the photosynthetic apparatus.

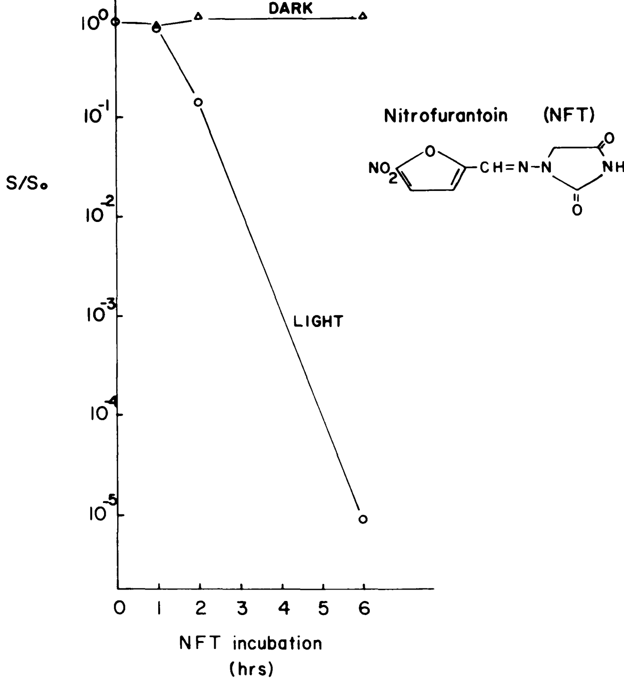

Figure 4. Effect of nitrofurantoin on the viability of A. nidulans. The experiment was performed as described in Figure 2.

Since our ultimate goal is to obtain mutants affecting most of the proteins in the photosynthetic membrane, we have continued to search for enriching agents. Our starting point has been the redox active compounds described by Biaglow et al. (5). We are searching for agents that have the same discriminatory abilities as metronidazole but that have different redox potentials. Such compounds should interact with different components in the electron transport chain, thus enriching for mutants in various chain segments. In particular, we would like to obtain an agent that could increase the probability of detecting mutants in the oxygen-evolving apparatus. One promising compound is nitrofurantoin, another antimicrobial agent with an $E_0' = -250mV$ (Figure 4). This drug kills A. nidulans in the light with kinetics almost identical to that of metronidazole under the same conditions. However, there was absolutely no killing in the dark for at least the first 6 hours. Nitrofurantoin appears to be another useful chemical enrichment agent; based on its redox potential, it might enrich for mutations in the electron transport chain that we cannot obtain with metronidazole (Figure 1).

TABLE II
Effect of Redox Active Compounds
on Anacystis nidulans Viability

Compound	Redox Potential	Survival (Light) / Survival (Dark)
3,6-dimethyl-p-benzoquinone (10μM)	+170mV [a]	Low
3,5-dinitrobenzonitrile (100μM)	-160mV [b]	High
Nitrofurantoin (1mM)	-250mV [b]	Low
Metronidazole (1mM)	-520mV [b]	Low
2-methyl-5-nitroimidazole (10μM - 1mM)	-560mV [b]	High

[a] Data from W. Oettmeier and W. Lockau (1973), Z. Naturforsch. 28C, 717-721.
[b] Data from J.E. Biaglow et al. (5).

A few of the other agents that we have screened are listed in Table II. We now have three compounds that are toxic to A. nidulans only when treated in the light. In particular, dimethylquinone may be valuable for the isolation of mutants in the electron transport chain between the two photosystems. In general, only about half of the compounds tested on A. nidulans kill in the light but not in the dark. However, none of these agents shows any discriminatory power on Aphanocapsa 6714 (Astier and Guikema, personal communication). The heterotrophic metabolism of this strain obviously complicates the cellular interactions of these drugs. Nonetheless, we plan to continue screening compounds in Aphanocapsa with the hope that its heterotrophic capabilities can be put to use in the study of photosynthesis.

Photosynehetic Mutants in Anacystis nidulans and Synechococcus cedrorum

We have characterized approximately 50 photosynthetic mutants in the two cyanobacterial strains isolated by the techniques described in the preceeding section. In addition, we have obtained pigment and herbicide-resistant mutants. Until we recognized the advantage of the transformable strain, A. nidulans R2, our wild-type strain was Synechococcus cedrorum. These two strains are very

TABLE III
Temperature-Sensitive Photosynthetic Mutants in Cyanobacteria

PSI

1) High F_v and F_{max}

2) Low F_v and F_{max} - High F716

3) High F_o and F_{max}; Low F_{max}^D/F_{max}

PSII

1) Low F_v, High F_{max}

   a) Low F696
   b) High F696

PSI + PSII

1) High F_o and F_{max}; Low F_{max}^D/F_{max}

Electron-Transport Components

1) High F_v and F_{max}

2) High F_v

3) High F_v, Low F_o and F_{max}; High F716

4) Low F_v and F_{max}

related (36), and they appear to have identical photosynthetic properties. The only differences between the two strains are transformability and a possible alteration in cell wall morphology. Fortunately, the enrichment procedures that we have developed work exceedingly well with A. nidulans and we now are characterizing the numerous mutants we have obtained in this strain. The mutants in S. cedrorum have provided us with a great deal of information on the structure and function of the photosynthetic membrane and we will continue analyzing them in detail. In this section, we will briefly discuss the type of mutants we have isolated and describe the properties of one well-characterized strain.

The data in Table III represents an outline of some temperature-sensitive, photosynthetic mutants obtained in the cyanobacteria. They are classified by two functional properties: defective photosystem activity and fluorescence characteristics. In total, these strains represent lesions in a minimum of 20 separate membrane proteins. Those mutants perturbed in PSI display a variety of fluorescence changes. Although the details of chlorophyll fluorescence analysis have not been discussed in this article, it is a rapid, highly-sensitive, and non-destructive technique for measuring photosynthetic function (25). Such measurements will be

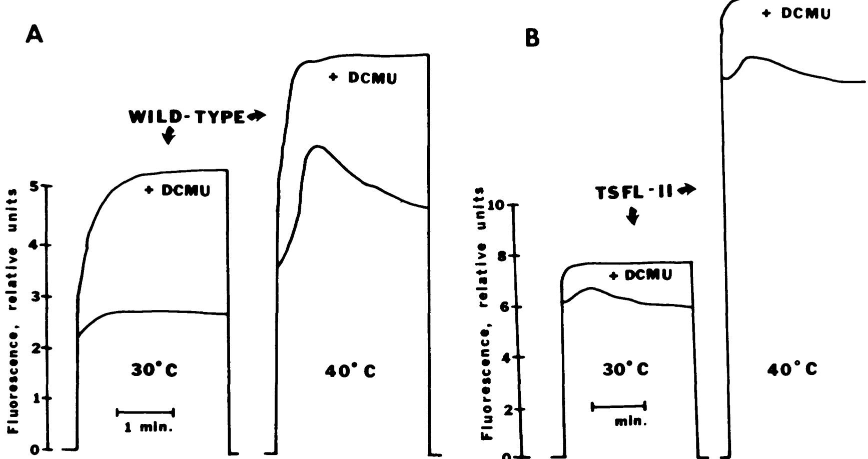

Figure 5. Chlorophyll fluorescence induction kinetics of S. cedrorum wild-type and mutant tsf1-11. Fluorescence was measured by illuminating a cell suspension containing 2ug chlorophyll/ml at an intensity of 5.6 mW/cm^2 through a 690nm interference filter. DCMU was added to a final concentration of 10^{-5}M (31).

extremely valuable in future cloning experiments. If a cloned fragment in a mutant has produced a functionally normal membrane, this can be seen readily by the restoration of normal fluorescence kinetics. A similar, rapid method for the detection of structural defects would also be valuable. Using the lithium dodecyl sulfate gel electrophoresis system of Delepelaire and Chua (10), we have developed a procedure to detect cytochromes (16) directly on acrylamide gels. This technique promises to be extremely useful in the characterization of specific mutations.

The properties of one high-fluorescence, temperature-sensitive mutant provides an interesting example of the type of analysis possible with these strains. The mutant, S. cedrorum tsf11-11, grows slowly at 40°C and has vastly reduced PSII activity at this temperature (30,31). The cells grown at 40°C elicit a very high level of fluorescence which is over 5.5 times the level of the wild-type grown at 30°C, and over 2.5-fold greater than the wild-type grown at 40°C. The variable fluorescence (the emission obtained after the first second of illumination) is much reduced, as is sensitivity to DCMU (Figure 5). All of these characteristics are typical of a lesion near PSII. The low-temperature fluorescence spectrum shows a major abnormality; there is little emission at 696nm (Figure 6). Emission at 696nm is though to be associated with PSII, although the physical nature of the emitting species is unknown. Therefore, all of the major functional assays indicated that tsf1-11 was due to a lesion that directly affects PSII.

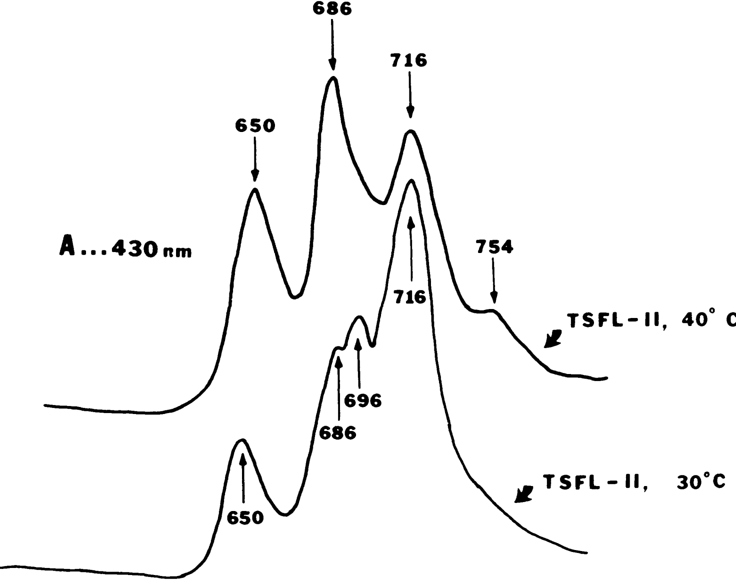

Figure 6. Liquid nitrogen fluorescence spectra of S. cedrorum grown at 30°C and 40°C. Cells at a concentration of 30ug chlorophyll/ml were excited with 430nm light (31).

Evidence that the lesion was a protein in the PSII complex was obtained by SDS gel electrophoresis of membranes and photosystem particles (Figure 7). The PSI and PSII complexes (23) are highly enriched in activity and show little or no cross-contamination. Electrophoresis of purified membranes indicated that a 53,000 dalton polypeptide was replaced by a 51,000 dalton species when tsfl-11 was grown at 40°C. PSII particles demonstrated the same change - PSII particles from cells grown at 40°C had a 51,000 dalton protein instead of the 53,000 dalton protein seen in 30°C-grown PSII particles (27). Therefore, we conclude that the 53,000 dalton protein is involved in the functioning of PSII. A slight modification of this polypeptide leads to aberrant PSII activity, high fluorescence, and a much depressed emission at 696nm. This is one of the few photosystem proteins to be characterized by a combined genetic, biophysical, and biochemical approach.

Transformation in Anacystis nidulans R2

The ability to use recombinant DNA technology in the cyanobacteria depends, in part, on a means of genetic exchange between

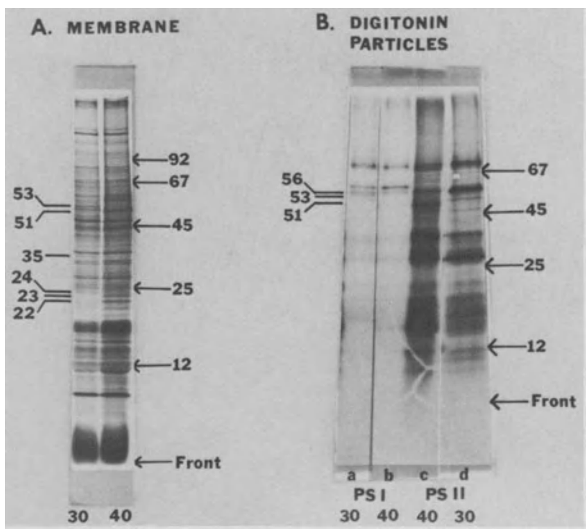

Figure 7. Polypeptide pattern on SDS polyacrylamide gels of S. cedrorum tsf1-11 membranes (A) and photosystem particles (B). A-^{35}S-labeled membrane proteins of tsf1-11 grown at either 30°C or 40°C. B-^{125}I-labeled photosystem particles from the mutant grown at the two temperatures. The protein changes in the 18-25 kilodalton range of the ^{35}S-labeled membranes were also found in wild type membranes used as a control and are not discussed further. The gels contain a 10-20% acrylamide gradient (27).

cells. Of the three types of genetic exchanges known in bacteria, only transformation has been conclusively demonstrated in the cyanobacteria. In the unicellular cyanobacteria, transformation has been reported in several strains (1,17,32,38), through the frequency of this event varies widely. Since we had been using Synechococcus cedrorum UTEX 1191 as our parent strain for the initial isolation of temperature-sensitive mutants, we analyzed this strain for transformability. We also tested one of Shestakov's transformable strains, A. nidulans R2, that was obtained from Shestakov by van den Hondel et al. (42). We were unable to transform S. cedrorum by either chromosomal or plasmid DNA. Attempts to induce transformation with modifications of the Ca^{+2} treatment (8) were unsuccessful as well. However, A. nidulans R2 showed a high frequency of transformation (Table IV,(42) and personal communication). This strain showed high transformation frequencies for both chromosomal and plasmid DNA, and these rates could not be increased substantially by Ca^{+2} treatment (Table IV). The cells are competent throughout the growth cycle and require no special treatment to induce transformability. A. nidulans R2 was therefore chosen as the host strain for the construction of the hybrid vectors to be discussed below, as well as

TABLE IV
Transformation of Cyanobacterial DNA into A. nidulans and S. cedrorum

Recipient strain + Donor DNA	Transformants/ml culture		
	+10mM NaCl	+30mM $CaCl_2$	+BG-11
A. nidulans R2 + 8.3 Mdal (Ap^{res})	980	960	620
S. cedrorum 1911 + 8.3 Mdal (Ap^{res})	0	0	0
A. nidulans R2 + 1191 Cm^{res}	1240	1210	850
S. cedrorum 1191 + 1191 Cm^{res}	2	0	1

Transformation was performed using 1 ml cell aliquots with either 1 μg DNA (8.3 Mdal) or 20 μg DNA (1191 Cm^{res}). Treatment with 10 mM NaCl and 30 mM $CaCl_2$ was based on the procedure of Cohen et al. (8). Cells were centrifuged and resuspended in 1/2 vol 10 mM NaCl. An aliquot was then used for transformation at 30°C. The remainder of the cells were then eventually resuspended in 1/10 vol 30 mM $CaCl_2$ and kept at 40°C for 30 min to 1 hr before transformation. The results are the average of three experiments with triplicate measurements and are normalized to the original cell concentration.

for all future genetic studies. As we mentioned previously, we have had no trouble in generating photosynthetic mutants in this strain.

Plasmids and Construction of Cloning Vectors

Evidence has accumulated recently that most strains of unicellular cyanobacteria contain at least two plasmids (19,41). The plasmids of A. nidulans R2 have molecular weights of 5.3 Mdal and 33 Mdal (42). Analysis with restriction enzymes has revealed that the 5.3 Mdal plasmid has only one cleavage site for either Xho I and Bam H1 and no sites for Eco R1 and Sal I. This plasmid has been detected in all tested strains except for a particular strain of A. nidulans which has been maintained at Dalhousie University for a number of years (19). A. nidulans R2 has also been cured of the 5.3 Mdal plasmid by artificial means (van den Hondel, personal communication). In both cases, the strains remain photosynthetically competent. Other attempts to determine the function of plasmid gene products have been unsuccessful; at the present time, both plasmids are genetically cryptic.

Because neither plasmid codes for a known genetic marker, it would be advantageous to produce a plasmid that contained a selectable marker such as antibiotic resistance. This approach was first accomplished by van den Hondel et al. (42), who transformed

A. nidulans R2 with the bacterial plasmid pR146. This plasmid contains the transposon Tn901 which carries the gene for ampicillin-resistance. Transposition of Tn901 into the 5.3 Mdal plasmid occurred, yielding an 8.3 Mdal plasmid that conferred ampicillin-resistance on the cell. This plasmid still has only one Xho I site, no Eco R1 sites, but now contains 2 Bam H1 sites. It was then possible to treat this plasmid with Bam H1, ligate the fragments, transform again into R2 and screen for ampicillin-resistant cells. Such endonuclease digestion yields two fragments of molecular weight 5.5 Mdal and 2.8 Mdal. It was found that the 5.5 Mdal fragment could be religated and replicate stably in R2. Though it confers a lower level of ampicillin resistance to the cells than the 8.3 Mdal plasmid, the 5.5 Mdal plasmid is still a usable cloning vector.

This procedure could be taken a step further if a hybrid between an E. coli plasmid and the 5.3 Mdal plasmid could be formed. Not only would this molecule be genetically marked, but it could also replicate in E. coli. However, numerous experiments in which bacterial plasmids without transposons were transformed into A. nidulans R2 were unsuccessful (Sherman; van den Hondel et al., unpublished observations). Thus, two different, but related, approaches were taken. In both cases a hybrid plasmid between an E. coli plasmid and the 5.3 Mdal cyanobacterial plasmid was constructed. The easiest solution to such a problem is to perform the construction in vitro - digest the plasmids with restriction enzymes, ligate the appropriate fragments, transform into E. coli, and select for appropriate recombinants. However, at the time these experiments were proposed, the containment level required for these experiments in The Netherlands were not available. We thus took an alternative approach and constructed a hybrid in vivo. First, a chloramphenicol-containing transposable element was added to pBR322 (7) and this new plasmid was transformed into A. nidulans R2. To select for formation of a cointegrate between the two plasmids we screened for ampicillin-resistant A. nidulans. Plasmids from ampicillin-resistance cyanobacteria were isolated and analyzed by transformation into E. coli and A. nidulans and by treatment with restriction enzymes.

The initial step in the in vivo procedure was to insert a chloramphenicol transposon into pBR322. We used an E. coli strain that contained a Cm element with a very high transposition frequency and that is presently under study (Sherman and van de Putte, unpublished observations). The transformation into A. nidulans R2 was performed with a molecule of pBR322:: Cm in which the transposon was inserted into pBR322 between the Bam H1 and Sal I sites in the tetracycline gene. Cointegrate formation between this plasmid and the indigenous 5.3 Mdal species was detected by selecting for A. nidulans colonies resistant to ampicillin. This should yield a hybrid plasmid containing the origin of replication of the 5.3 Mdal plasmid, the ampicillin gene of pBR322 and, hopefully, the BR322 origin. A diagrammatic representation of the hybrid construction is

TABLE V
Transformation of Hybrid Plasmid DNA, pLS103, into A. nidulans and E. coli

Plasmid Donor DNA	Recipient	No. Ap^{res} Transformants/ml/µg DNA	Recipient	No. transformants/ml/µg
R2(pLS103)	A. nidulans R2	135 R2$(pLS103)_2$	R2	125
			HB101	715
	E. coli HB101	725 HB101$(pLS103)_1$	R2	120
			HB101	675
8.3 Mdal	A. nidulans R2	225	R2	270
pBR322	E. coli HB101	1650	HB101	1530

Transformation and DNA isolation were performed as described in Materials and Methods with 0.7 to 1.0 µg/ml DNA. All results were normalized to transformations/µg DNA and to original cellular concentration. Results are each the average of two experiments done in triplicate. Ampicillin resistance was monitored on plates with 0.5 µg/ml Ap for A. nidulans and 40 µg/ml Ap for E. coli.

shown in Figure 8a. Six ampicillin-resistant colonies were obtained from this experiment and the plasmids were designated pLS101 - pLS106. Plasmid DNA was isolated from each strain and analyzed by agarose gel electrophoresis. The plasmids range in size from 6.0 Mdal for pLS104 to 14.0 Mdal for pLS105. None of the strains contained the 5.3 Mdal plasmid, but all still had the 33 Mdal species. Since the strain containing pLS103 grew best on ampicillin and since this plasmid had a relatively small size (6.8 Mdal), it was chosen for more detailed study.

Transformation into A. nidulans and E. coli with pLS103

The hybrid plasmid pLS103 was isolated from A. nidulans R2 and transformed into E. coli HB101 (recA hsr^-, hsm^-). As shown in Table V, this DNA could confer ampicillin resistance on both strains with an efficiency comparable to that of standard plasmid DNA controls. This implies that pLS103 contains the replication origin of both pBR322 and the 5.3 Mdal plasmid. In addition, pLS103 could be amplified in E. coli with chloramphenicol and spectinomycin; however, we have not been able to demonstrate amplification in A. nidulans.

After this first cycle of transformation, one colony of ampicillin-resistant E. coli (HB101 (pLS103)), and A. nidulans (R2 $(pLS103)_2$) was picked, grown in liquid medium and the plasmids from each strain were isolated. The DNA was again transformed into the two strains, as shown in Table V. Transformation frequencies were quite similar to those obtained with the original pLS103 isolated from A. nidulans. Thus, biological activity was not modified by

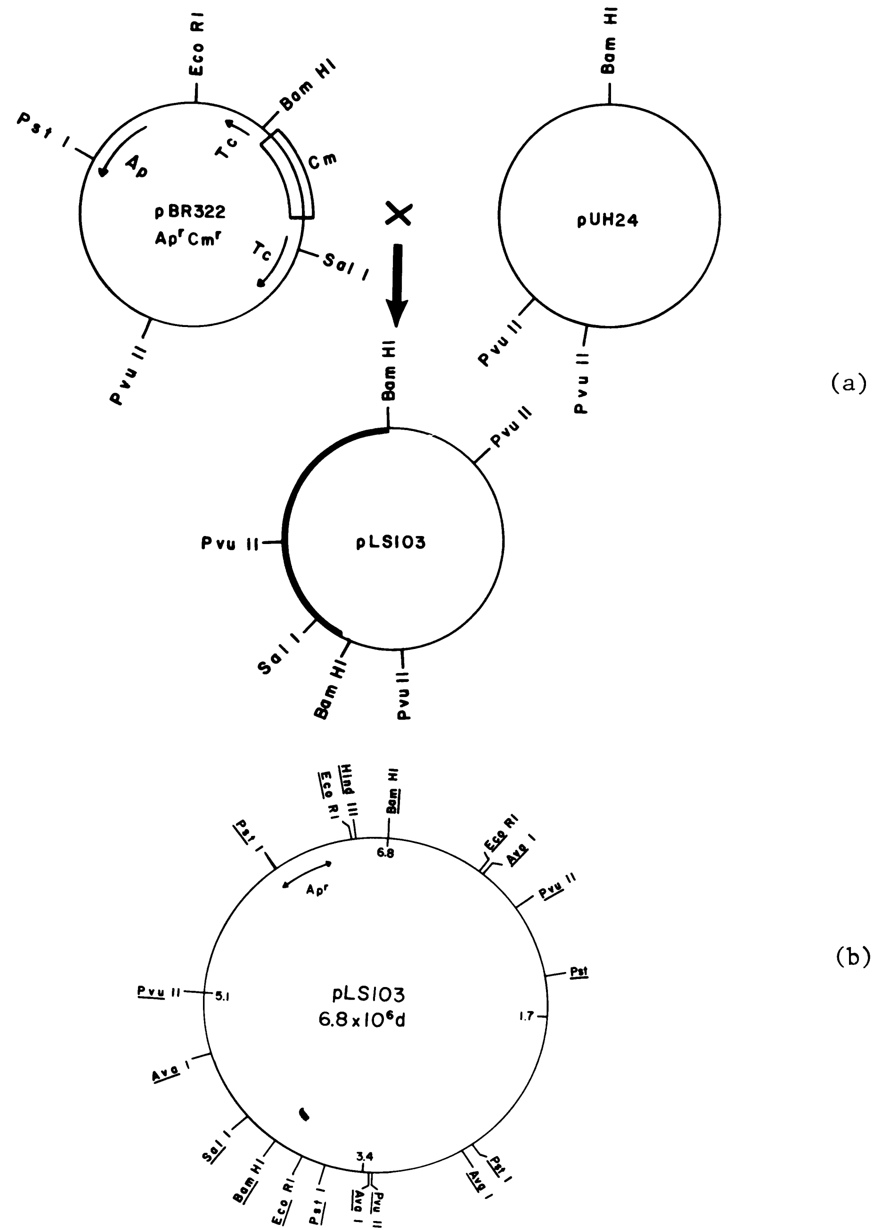

Figure 8. (a) Diagrammatic representation of the construction of pLS103 in vivo. The cyanobacterium A. nidulans was transformed with pBR322:: Cm and Ap^r cells were selected. Cointegrate formation between pBR322:: Cm and the indigenous plasmid pUH24 (5.3 Mdal) resulted in the formation of the hybrid pLS103. The heavy line in the hybrid represents

growth in heterologous bacteria. The restriction endonuclease cleavage patterns of pLS103, R2(pLS103)$_2$, or HBlol(pLS103) were also identical; the DNA is thus apparently not changed during growth in different organisms. Because the DNA could be amplified in *E. coli*, plasmids from this source were used for physical characterization. This source of the plasmid is preferable since it could be isolated easily with no contamination from the 33 Mdal species.

The molecular weight of the hybrid plasmid was determined by the usual techniques of agarose gel electrophoresis in conjuction with digestion by restriction endonucleases. As shown in Figure 8b, pLS103 has a molecular weight of 6.8 Mdal. This value was obtained after multiple digests of the plasmid; the fragments produced by selected endonuclease cleavage is shown in Figure 9, and the size of the fragments is listed in Table VI. Since the size of the plasmid is smaller than the sum of the sizes of pBR322, 5.3 Mdal and the transposon, it is obvious that parts of these molecules were deleted after cointegrate formation. The deletion of a number of restriction sites can be seen from the data in Table VII. For example, 2 *Bgl* II sites, 2 *Hind* III sites, 3 *Kpn* I sites, and the unique *Xho* I site have been deleted. Interestingly, most of the deleted pieces of DNA derive from the two large segments of the 5.3 Mdal plasmid (42). The pLS103 hybrid retains the unique *Sal* I site from pBR322, and one *Bam* H1 site from each original molecule. The hybrid has three *Eco* R1 sites, implying that there may be vestigial fragments of the transposon remaining. The map indicates that the site of transposition was between the two *Pvu* III sites on the 5.3 Mdal plasmid. Based on the transpositions generated by van den Hondel *et al*. (42) and the deletions obtained in pLS103, it is probable that the replication origin of 5.3 Mdal is near the *Bam* H1 site (Sherman and van de Putte, manuscript in preparation).

The construction of pLS103 was performed in order to obtain a vector that could be used for the cloning of photosynthetic genes in *A. nidulans*. This is the second example of such a vector constructed *in vivo* using bacterial transposons (42). The advantage of the present vector is readily apparent. The plasmid is small and can be grown easily in *E. coli*. Therefore, isolation of large quantities of the plasmid and the cloned DNA will be much easier than if *A. nidulans* was used as the host. Moreover, the ability of the plasmid to be grown in *E. coli* facilitates the insertion into the vector of transposons containing additional antibiotic markers. This will permit us to use the powerful technique of insertional inactivation (40). Finally, it is now possible to clone *E. coli* genes on this

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the position of pBR322. (b) The circular restriction map of pLS103. Locations of the restriction cleavage sites were determined by digesting the plasmid with one or more restriction enzymes and analyzing the size and number of the fragments produced.

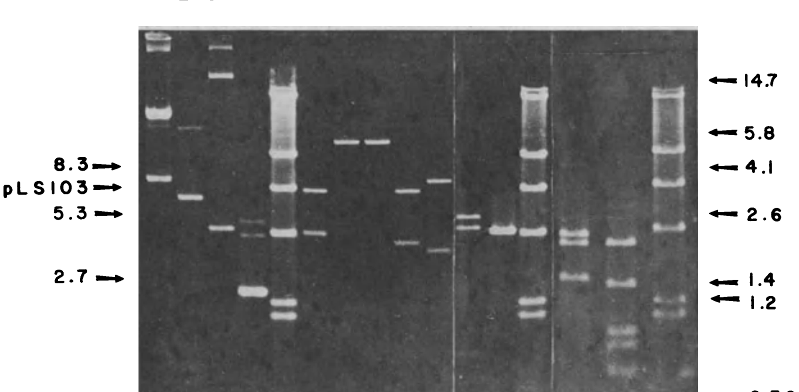

Figure 9. Agarose gel electrophoresis of plasmid pLS103 and various restriction digests. Electrophoresis was performed with samples containing approximately 1µg DNA on 0.7% agarose gel at 30V for 16 hr. The samples are arranged as follows: Slots 1-4, undigested plasmid DNA and associated markers; and slots 5-16, restriction digests of pLS103 with λ*Hind* III digests as references. Slot 1 - Plasmid pCH1 (8.3 Mdal) from van den Hondel *et al*. (42); Slot 2 - pLS103; Slot 3 - pUC24 (5.3 Mdal) and the 33 Mdal *A. nidulans* plasmid; Slot 4 - pBR322. The plasmid form indicated by the arrow and molecular weight in each slot in this figure is the covalently closed circular form as determined by the heat treatment procedure of van den Hondel *et al*. (40) (data not shown). Slot 5 - *Hind* III fragments of phage λ. The molecular weights of the bands from top to bottom are: 14.7 5.8, 4.1, 2.6, 1.4, 1.2, and 0.36 Mdal. Slot 6 - pLS103 *Bam* H1; Slot 7 - pLS103 *Hind* III; Slot 8 - pLS103 *Sal* I; Slot 9 - pLS103 *Hind* III + *Bam* H1; a 0.2 Mdal fragment is not visible in this gel; Slot 10 - pLS103 *Hind* III + *Sal* I; Slot 11 - pLS103 *Eco* R1; Slot 12 - pBR322 *Eco* R1; Slot 13 - λ*Hind* III; Slot 14 - pLS103 *Pvu* II; Slot 15 - pLS103 *Pvu* II + *Bam* H1; Slot 16 - λ*Hind* III.

plasmid and study the expression of these genes in an *A. nidulans* background. Importantly, the construction of pLS103 demonstrates that *E. coli* and *A. nidulans* hybrid plasmids can be formed and stably maintained.

We can now extend this approach by constructing specific hybrids *in vitro*. These hybrids are now being produced in our lab and in the laboratory of van Arkel and van den Hondel (personal communication). An example of one such hybrid that we are now studying is depicted in Figure 10. In this case, we have used the plasmid pBR328 (33) which codes for antibiotic resistance to ampicillin, chloramphenicol, and tetracycline. Both the 5.3 Mdal plasmid and pBR328 were cut with *Bam* H1, and treated with bacterial alkaline

TABLE VI
Restriction-Endonuclease Cleavage Fragments of pLS103 (in Mdal)

1. Bam H1 - 4.0; 2.8
2. Hind III - 6.8
3. Sal I - 6.8
4. Hind III + Bam H1 - 4.0; 2.6; 0.2
5. Hind III + Sal I - 4.4; 2.4
6. Pvu II - 2.7; 2.4; 1.7
7. Eco R1 - 3.2; 2.8; 0.8
8. Pvu II + Bam H1 - 2.4; 1.7; 1.1; 0.9; 0.73
9. Pst I - 2.4; 2.1; 1.25; 0.95
10. Pst I + Bam H1 - 2.1; 1.45; 1.25; 0.95; 0.65; 0.35

TABLE VII
Number of Restriction Enzyme Cleavage Sites in Plasmid pLS103

Enzyme	pLS103	pBR322[a]	5.3 Mdal (pUH24)[b]
Ava I	4	1	3
Bam H1	2	1	1
Bgl II	0	0	2
Eco R1	3	1	0
Hind III	1	1	2
Kpn I	0	0	3
Pst I	5	1	7
Pvu II	3	1	2
Sal I	1	1	0
XhO I	0	0	1

[a] Data from Sutcliffe (39).
[b] Data from this communication and van den Hondel et al. (40, 41).

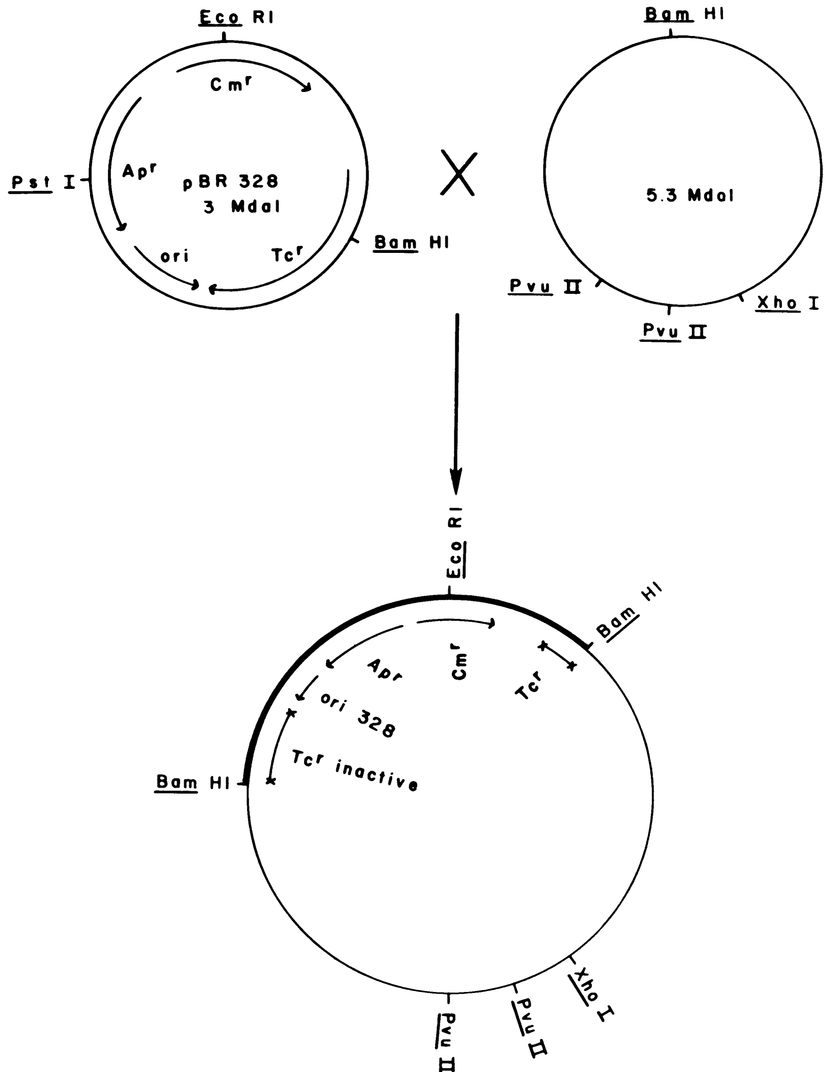

Figure 10. Diagrammatic representation of the construction of a hybrid plasmid _in vitro_. The _E. coli_ plasmid pBR328 and the _A. nidulans_ 5.3 Mdal plasmid were each digested with _Bam_ H1, religated and transformed into _E. coli_. Ampicillin- and cloramphenicol-resistant, but tetracycline-sensitive colonies were selected; plasmids were then isolated and transformed into _A. nidulans_. The heavy line represents the position of pBR328.

phosphatase; the two DNAs were then mixed and ligated. After transformation into E. coli, ampicillin-resistant and tetracycline-sensitive colonies were selected. Plasmid DNA from these strains was then isolated and measured; those of the appropriate size (about 8.3 Mdal) were transformed into A. nidulans to ensure proper replication in the cyanobacterium. This hybrid represents an ideal cloning vector since it still contains two functional genes coding for antibiotic resistance. The inactivation of the tetracycline gene is of no consequence, because the light-sensitivity of this drug makes it inappropriate for use with photoautotrophs such as cyanobacteria. One benefit of this hybrid is the fact that it possesses a unique Eco R1 site in the chloramphenicol gene that can be used for cloning via insertional inactivation. It is thus obvious that numerous hybrid plasmids can be constructed to allow the use of different restriction enzymes in cloning experiments.

Cloning Experiments

We now have photosynthetic mutants, transformation, and cloning vectors in the cyanobacterium Anacystis nidulans R2. Therefore, we have the ability to select for cloned genes directly. Though this selection may be a tedious project, we do not anticipate substantial difficulties. We will transform plasmids containing chromosomal DNA into a mutant and screen for colonies that can grow on ampicillin at 40°C. This is a powerful screening technique, and we should be able to detect the rare clone containing the appropriate gene fragment. The ultimate benefit of this system is that we have the potential to clone many genes coding for photosynthetic functions without having prior knowledge of the gene product.

Once we determine that a mutant phenotype can be reversed by the presence of a recombinant plasmid, the photosynthetic properties of the cloned DNA will be studied. We will synthesize the gene product either in vitro or using E. coli maxi- or mini-cells. We will thus be able to identify a particular protein with a specific function. The ability to synthesize large quantities of a particular gene product will open up new vistas in photosynthetic research, especially functional manipulation in vitro. For example, it should be possible to use either model membranes or stripped photosynthetic membranes and add the purified proteins to them singly or in combinations. This will allow the study of the precise functional characteristics of each membrane component. It is obvious that the potential of this system for the analysis of photosynthetic membrane assembly is virtually unlimited.

The ultimate benefits of the cyanobacterial cloning system are profound and go far beyond the immediate aims stated above. For example, there may be some near-term practical benefits such as the cloning of appropriate genes to convert A. nidulans into a

heterotroph. As mentioned earlier, this will greatly aid in the search for mutants. Longer term benefits can be even more striking. Numerous genes could be cloned into A. nidulans and their proteins produced using only light as an energy source. The examples that could be listed are endless, but one field has been mentioned by numerous observers--the cyanobacteria might eventually be used as energy factories capable of converting light into directly useable energy sources such as hydrogen or hydrocarbons. Though such applied results are years away, they lend emphasis to our intial efforts in establishing the overall system.

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DISCUSSION

Q. MACRINA: Have you performed a competency curve using the R2 strains?

A. SHERMAN: This has been done by van den Hondel and van Arkel of the University of Utrecht. They found little difference in transformation during the growth cycle, although the maximum rates are obtained during mid-log phase.

Q. MACRINA: Will culture supernatant from the R2 strain convert a non-transforming *A. nidulans* strain to one that now transforms?

A. SHERMAN: No. This experiment has been tried many times with no success.

MODELS FOR GENETIC MANIPULATION OF ACTINOMYCETES

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INTRODUCTION

The actinomycetes comprise a large diverse group of mycelial bacteria of both economic and medical importance. More than three thousand antibiotics, including most of those of clinical utility, are produced by this group of organisms. While thirty-four genera of actinomycetes are known to elaborate antimicrobial substances, the majority of antibiotics are produced by the genera *Micromonospora*, *Nocardia*, *Streptoverticillium*, *Streptosporangium*, and *Streptomyces*. Over 60% of known antibiotices are produced by the latter genus (3) in which more than 900 antibiotic-producing species have been described. Until recently, genetic exchange has been difficult to demonstrate in the actinomycetes. There has thus been little practical application of directed genetic recombination in this group. With the development of methodologies and techniques for DNA cloning in actinomycetes however, our ability to genetically manipulate these industrially important microorganisms should increase dramatically.

DISCUSSION

I. Mechanisms for Genetic Exchange in Actinomycetes

Conjugation. Before the advent of *in vitro* recombinant DNA technology and protoplast fusion techniques, the method used most frequently for effecting genetic exchange in streptomycetes was conjugation. A number of species of *Streptomyces* have been examined by conjugational analysis and several presumptive linkage maps have been constructed (1,10). However, because recombination is difficult to

demonstrate in many strains of streptomycetes - especially those of industrial importance - conjugation has not been of great utility in bringing about directed genetic exchange among streptomycetes.

Protoplast fusion. In 1977, Hopwood *et al.* (15) described a protoplast fusion technique for streptomycetes. They predicted useful application of the technique both in facilitating interspecific crosses of streptomycetes and in greatly increasing recombination frequencies in intraspecific crosses. It was suggested that applications would lead to the discovery of new antibiotics and the construction of high-titer antibiotic-producing strains. Although both of these goals may be achievable employing protoplast fusions, there are drawbacks to this approach. Successful interspecific recombination assumes sufficient DNA homology between the strains being crossed for homologous recombinational events to occur. Such homology may not exist between strains in a desired cross. If the parents in such interspecific crosses are antibiotic producers, the detection and isolation of a new antibiotic activity may be difficult - requiring the elaborate identification and isolation procedures presently used in antibiotic discovery programs. Fusion techniques may be of greater utility in intraspecific crosses to create high-titer antibiotic-producing strains of streptomycetes - especially if coupled to current mutation-selection programs. Protoplast fusion however may be of greatest benefit in facilitating rapid genetic mapping of streptomycetes - providing information of value for cloning streptomycete genes. Baltz (2) and Ochi and Katz (26) have recently used this technique to generate map data in *Streptomyces fradiae* and *S. parvulus*. Recombination frequencies in these crosses were very high, ranging from 0.5 to 15%.

Transduction-Transformation. Transduction and transformation processes have been of limited value in effecting chromosomal genetic exchange in actinomycetes. A transformation system was described by Hopwood and Wright (14) for *Thermoactinomyces vulgaris* but attempts to use transformation for genetic analysis in other actinomycetes have not been successful. Employing phage mediated transduction in actinomycetes, Stuttard (32) transduced several auxotrophic strains of the chloramphenicol producer *Streptomyces sp.* 3022a (a *Streptomyces venezuelae*) to prototrophy using the virulent phage ϕSV1. ϕSV1 has a very narrow host range which includes only *Streptomyces sp.* 3022a strains and some strains of *S. venezuelae*. The efficiency of transduction reported was 5-16 x 10^{-7} per phage DNA molecule. A recent paper by Mkrtumyan (23) also reports transduction in a streptomycete. Determinants located on the SCP2 plasmid of *S. coelicolor* were transduced to an SCP2$^-$ strain with the temperate phage VP5.

TABLE I
Plasmids Isolated from Actinomycetes

Strain	Antibiotics Produced	Plasmid	Mol. Wt. (X 10^{-6})	Reference
Streptomyces coelicolor A3(2)	methylenomycin A actinorhodin	SCP2 SCP1	18-20.0 -	(4,29,30)
Streptomyces erythreus UC 2017	erythromycin	pUC10	~7.0	(21)
Streptomyces espinosus	streptothricin	pUC6	~6.0	(22)
Streptomyces fradiae UC 2046	neomycin complex	pUC1 pUC2	~50.0 ~30.0	(9)
Streptomyces fradiae 676	neomycin	--	59.0	(3)
Streptomyces fradiae KCC S-0579 ATCC 10745	neomycin	large small	62.0 43.0	(27)
Streptomyces hygroscopicus 434	geldanamycin	pSHY1	37.9	(12)
Streptomyces kasugaensis MB 273	kasugamycin aureothricin	pSK1	6.7	(27)
Streptomyces lividans JI1326	--	SLP1.1-6	6.2-8.2	(7)
Streptomyces lavendulae KCC-50985 (S985)	streptothricin complex	pSL1	2.66	(24)
Streptomyces niveus KCC S-0599 NRRL 2466, UC 2094	novobiocin	pSN1	20.0	(27)
Streptomyces omiyaensis NIHJ AT-95	chloramphenicol	pSO1	19.0	(27)
Streptomyces puniceus KCC-S-0406	viomycin	pSPU1 pSPU2	17.3 39.1	(12)
Streptomyces ribosidificus KCC S-0923	ribostamycin	pSR1	~52	(27)
Streptomyces ribosidificus ATCC 21294	ribostamycin	--	52.0	(25)
Streptomyces rimosus 907	oxytetracycline	--	37.0	(3)
Streptomyces sp. 3022a (Streptomyces venezuelae) UC2374	chloramphenicol	pUC3	~20	(20)
Streptomyces violaceoruber SANK 95570	methylenomycin A	pSV1	100.0	(27)
Streptomyces sp. 2217-G_1	cycloheximide	pSCY1 pSCY2	3.2 18.0	(12)
Streptomyces sp. 7068-CC_1	neomycin	pSNE1 pSNE2	22.2 47.0	(12)
Streptomyces sp. 7434-AN_4	lankacidins	pSLA2	11.2	(13)
Streptoverticillium mashuensis KCC S-0059	streptomycin polyene antibiotic	pSM1	16.0	(27)

II. DNA Cloning in Streptomyces

Plasmid Vectors. In the last few years, a fairly large number of actinomycete plasmids have been isolated and studied. As seen in Table I, the hosts for these plasmids represent a diverse group of streptomycetes including producers of several different antibiotics. While the majority of the streptomycete plasmids isolated thus far have molecular weights of 20-100 x 10^6, a few are quite small and appear to be good candidates for cloning vectors. For example, pUC6 is a high copy-number cryptic plasmid (30-40 copies per chromosome) isolated from *Streptomyces espinosus* (22). It has a molecule weight of 6.0 x 10^6 and is easily isolated in good yield. It has a single Bgl II site which has been used to construct a pBR322/pUC6 cointegrate plasmid. With the addition of markers selectable in streptomycete hosts, pUC6 might serve as a useful bifunctional cloning vector. The ability to clone the plasmid intact in *E. coli* has allowed the deletion of several restriction fragments from the pUC6/pBR322 hybrid. pSK1, a plasmid isolated from *S. kasugaensis* and pSL1, isolated from *S. lavendulae* may also have potential as cloning vectors (24,27). pSL1 is a very small cryptic plasmid (m.W.=2.66 x 10^6) which is also reported to be present in high copy-number and has single restriction sites for Eco R1 and Sal I.

Two plasmids that have already been employed successfully as streptomycete cloning vectors are SCP2 from *S. coelicolor* (M.W.= 18-20 x 10^6) and SLP1.2 from *S. lividans* (M.W.=8.2 x 10^6) (5,7). Both plasmids can be isolated in good yield. SLP1.2 has a copy-number of 4-5. Although both plasmids are cryptic, their presence in a transformed recipient can be detected through their ability to produce "pocks." Colonies originated from spores carrying either SCP2 or SLP1.2 produce narrow zones of inhibition, "pocks," when plated on lawns of $SCP2^-$ or $SLP1.2^-$ strains. This process, termed "lethal zygosis", does not involve antibiosis but does require hyphal contact and may represent a stage in conjugation. The ability of these plasmids to produce pocks was used advantageously by Bibb, *et al.* (6) to develop an efficient polyethylene glycol assisted transformation procedure for streptomycete protoplasts. Utilizing this procedure, Bibb, Schottel and Cohen (5) successfully transfected the cloned gene for methylenomycin A resistance (Mm^r) into *S. lividans* using pSLP1.2 as vector (Figure 1). Methylenomycin A is an antibiotic produced by strains of *S. coelicolor* carrying the large fertility plasmid SCP1. The antibiotic inhibits some streptomycetes and several Gram-positive eubacteria. The SCP1 plasmid codes for both the methylenomycin A biosynthetic enzymes and for methylenomycin A resistance. Using an $SCP1^+$ source of donor DNA and an $SCP1^-$ *S. lividans* recipient, clones carrying SLP1.2 plasmids containing Mm^r genes were selected directly with methylenomycin A. The resultant hybrid Mm^r plasmid is designated pSLP111. Cohen (11) recently described a bifunctional plasmid, pSLP120, which was constructed by fusing pSLP111 and the *E. coli* plasmid pACYC184. pSLP120 replicates in both *E. coli* and *S. lividans*.

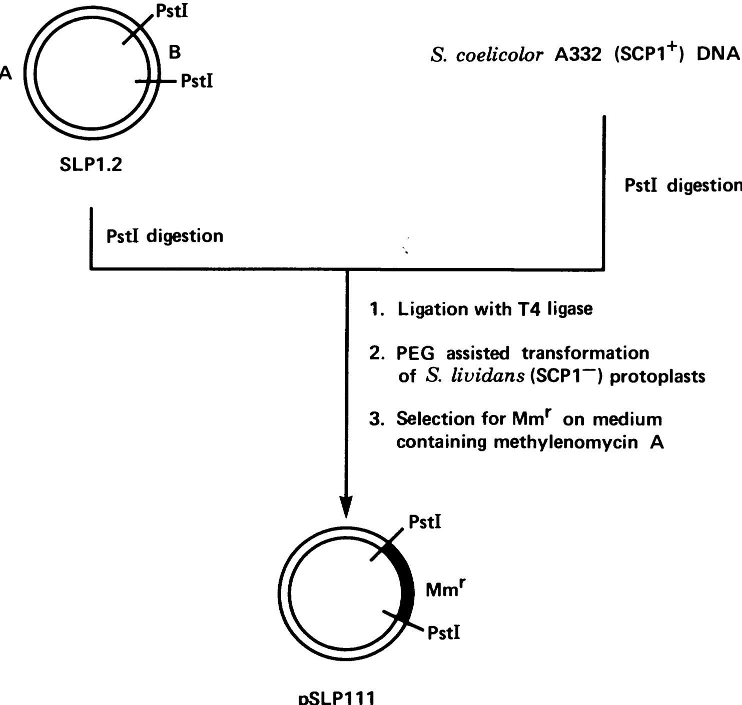

Figure 1. Cloning of Methylenomycin A resistance in Streptomyces lividans using SLP1.2 as vector.

Thompson et al. (35) have also reported success in cloning streptomycete genes using the SLP1.2 plasmid in S. lividans. The genes for neomycin phosphotransferase (APH) and neomycin acetyltransferase (AAC) were cloned from Streptomyces fradiae and introduced into S. lividans. The gene for an S. azureus ribosomal RNA methylase which determines resistance to the antibiotic thiostrepton was also cloned onto the SLP1.2 plasmid. In addition to the above resistance markers, Thompson et al. (35) report the successful cloning of two genes for amino acid biosynthesis from S. coelicolor A3 (2).

Actinophage Vectors. Actinophage are very prevalent in soils and are easily detected and isolated by plating soil extracts on soft agar overlays containing spores of streptomycete indicator strains. A large number of both virulent and temperate actinophages have been described (8,18). Temperate actinophages are being examined by several investigators for their potential use in cloning host-vector systems. Temperate phages studied in some detail include ϕC31, SH10 and R4 isolated from S. coelicolor, S. lividans, and S. parvulus respectively. To date, C31 has been studied most extensively. A partial genetic map of C31 has been constructed

and conventional conjugation mapping studies have identified the probable integration site of the phage in the *S. coelicolor* genome (18).

ϕC31 is a 37.7 kb linear actinophage with cohesive ends. The method of DNA packaging in this phage appears to be the same as that in the lambdoid coliphages. As a vector for DNA cloning, ϕC31 has the above advantage of available genetic information plus the advantages of a fairly broad host range and viable deletion mutants to allow DNA insertion (19). An *in vitro* packaging system may also be possible. One disadvantage of ϕC31 for cloning purposes is the absence of single sites for restriction enzymes. At present, partial digestions with enzymes which cleave the phage into 3-7 fragments must be employed for construction of DNA insertions.

Critical to the development of a phage cloning vector for actinomycetes was the development of an effective transfection system. As early as 1966, Okanishi *et al.* (28) successfully transfected *Streptomyces kanamyceticus* protoplasts with the phage PK-66. However, this system exhibited low transfection frequencies. Following the development of methodology by Bibb *et al.* (36) for the high frequency transformation of streptomycete protoplasts in the presence of polyethylene glycol, more efficient transfection procedures for actinomycetes were developed (16,17,33). These are now adequate for phage vector cloning systems. Transfection mixtures are plated in a protoplast-containing soft agar overlay on a protoplast regeneration medium agar base. Plaques generally become visible in 1-2 days.

A bifunctional replicon composed of pBR322 and ϕC31 has been described in Suarez and Chater (34) (Figure 2). Their work with the pBR322/ϕC31 replicon demonstrates that streptomycete DNA can be replicated and maintained in *E. coli* without loss of function and that, although a modest loss of infectivity occurs after growth in *E. coli*, no severe restriction barrier is operative in *E. coli* to *S. lividans* transfers.

CONCLUSIONS

The greatest impediment to date in applying recombinant DNA techniques to the actinomycetes has been the absence of suitable selective markers for cloning vectors. With the recently demonstrated ability to clone streptomycete antibiotic resistance genes and genes for amino acid biosynthetic enzymes onto streptomycete vectors, this hurdle has been overcome. The construction of useful vectors from existing or newly discovered actinomycete plasmids or phages should now be relatively straightforward. It is probable that both phages and plasmids will be employed in actinomycete cloning systems, the choice being dependent upon availability of a vector with the ability to replicate in the chosen recipient host. A truly broad host-range vector is not now available in the actinomycetes.

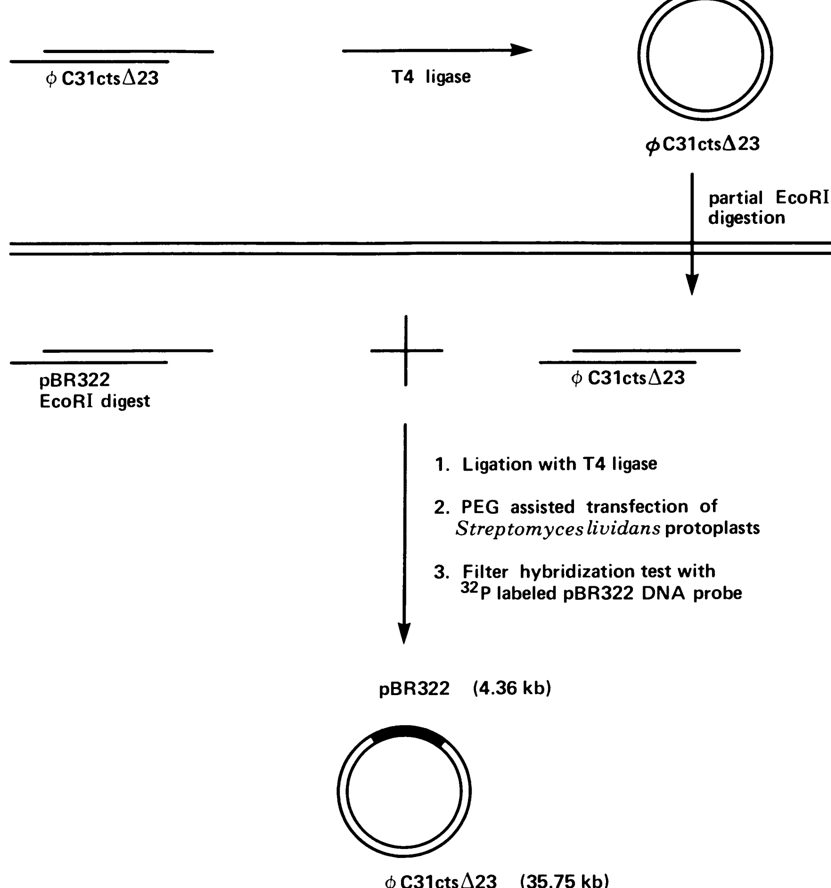

Figure 2. Construction of pBR322/ϕC31 bifunctional replicon.

Actinophages may have advantages over actinomycete plasmids as vectors for some cloning applications. With the use of a phage vector, plaque hybridization studies with radioactive probes become possible, thus facilitating actinomycete gene bank screening. The actinophages also generally have wider host ranges than those of actinomycete plasmids. Efficient phage promoters may also enhance expression of cloned genes in actinomycetes. *In vitro* engineering of lytic actinophage to essentially create small plasmid vectors containing the phage origin of replication may be an alternative for cloning in strains where plasmid or temperate actinophage vectors are not available.

In an industrial setting, and especially in the antibiotics industry, the application of recombinant DNA cloning techniques to actinomycetes holds much promise. For example, an increase in antibiotic titer in a fermentation might be achieved through insertion

of either a gene for a more efficient enzyme or more copies of a gene for a rate limiting enzyme in antibiotic biosynthesis. Insertion of genes which would enable an antibiotic producing actinomycete to utilize an inexpensive substrate for growth and antibiotic production also becomes a possibility. The in vivo modification of antibiotics in the producing organisms by addition of genes encoding enzymes from other organsims can also be envisioned as a means to produce a new or improved antibiotic.

Finally, although model host-vector systems now exist for DNA cloning in actinomycetes, even more rapid advances in our understanding of this group should follow the development of a vector allowing the expression of actinomycete DNA in E. coli. Availability of such a vector should enable the actinomycete geneticist to utilize the large number of available E. coli mutants for complementation studies, thus greatly facilitating the study and manipulation of actinomycete genomes.

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THE REGULATION OF YEAST GENE EXPRESSION BY MULTIPLE CONTROL ELEMENTS

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The effective employment of biological systems for production of commercially useful chemicals often requires a thorough understanding of how the desired system is regulated. In the past we have looked to procaryotic systems for models of how control is exerted; they have served us well. However, it is becoming abundantly clear that the procaryotic paradigm does not extend to eucaryotic systems. For these, new models must be developed and new paradigms constructed. My purpose is the development of such a model in the simple eucaryotic microorganism, *Saccharomyces cerevisiae*. This yeast is perhaps the oldest commercially cultured organism in existence. It is also one of the most well studied genetic systems available. Our model is the metabolism of two main nitrogen sources, allantoin and arginine. We selected two systems instead of one to gain some insight into the spectrum of regulatory mechanisms that operate in eucaryotic cells. Our choice also presents the added opportunity of assessing the means used to integrate the expression of separately controlled, but functionally related genes.

ALLANTOIN DEGRADATION

Allantoin, a product of adenine and guanine catabolism in many organisms, can serve as sole nitrogen source for *S. cerevisiae*. The degradation of allantoin involves five enzymatic steps and four transport systems (Figure 1). The hydantoin ring of the allantoin molecule is first opened in a hydrolytic reaction catalysed by allantoinase (16a, 29). Allantoate, the product of this reaction is degraded by allantoicase yielding one molecule each of urea and ureido-glycollate (27). Ureidoglycollate hydrolase then catalyses

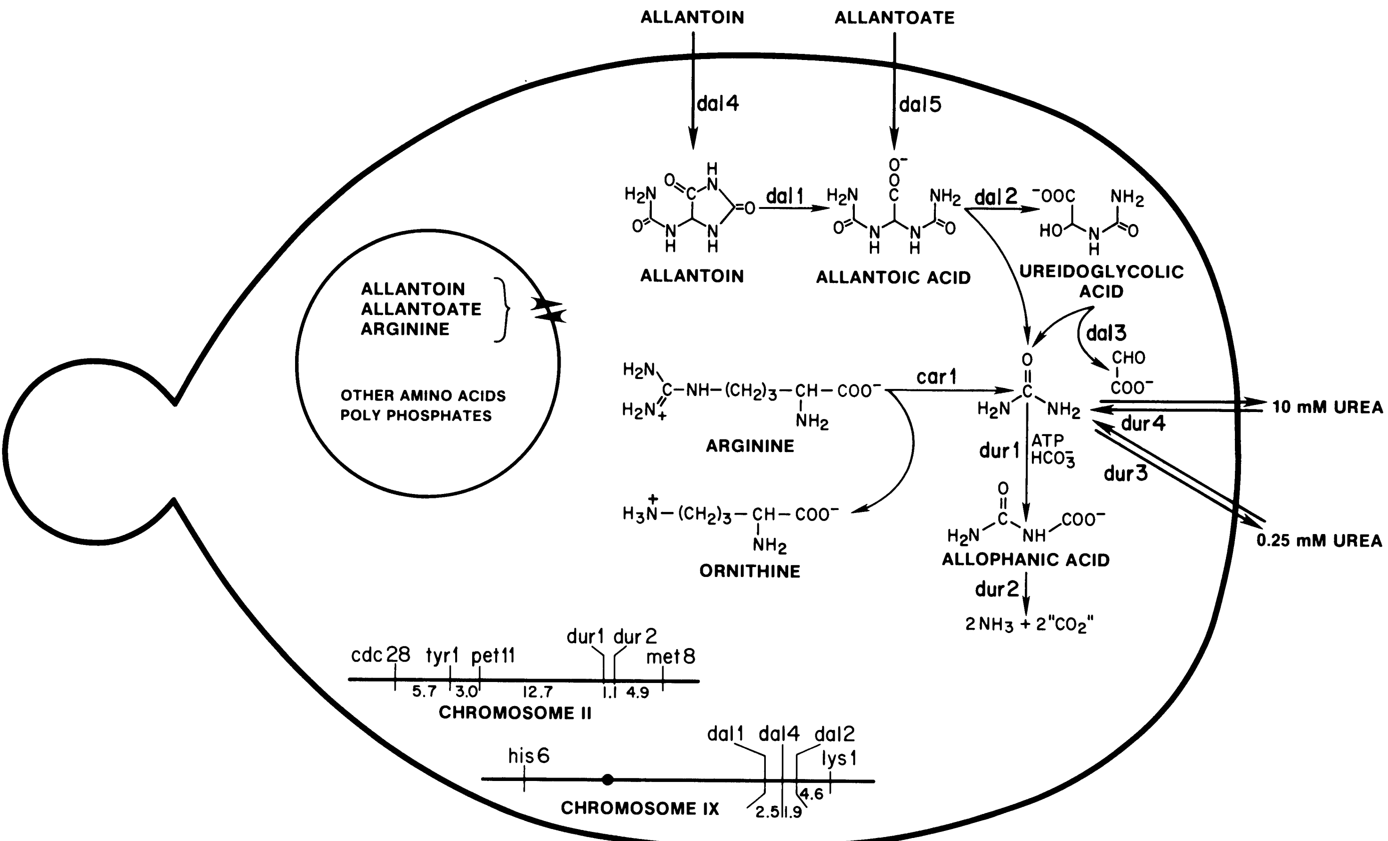

Figure 1. The enzymatic reactions and transport systems involved in the degradation of allantoin and arginine. The structural genes responsible for production of these components are indicated along with their genetic organization (lower left). The intracellular sequestration of nitrogen sources within the vacuole is also shown.

the hydrolytic cleavage of ureidoglycollate to glyoxylate and a second molecule of urea (10). In most organisms, urea is degraded to ammonia by action of urease. S. cerevisiae, however, does not contain demonstrable urease. Roon and Levenberg (20) reported an alternative mode of urea degradation in Chlorella and C. utilis that was inhibited by avidin. The reaction they reported is as follows:

$$\text{UREA} + \text{ATP} \xrightarrow{Mg^{++}\ K^{+}} CO_2 + 2\ NH_3 + \text{ADP} + P_i$$

We were able to demonstrate a similar activity in S. cerevisiae (31). Although the avidin sensitivity of this reaction indicated a role for biotin, it was difficult at the time to see how biotin participated as a prosthetic group. Therefore, we isolated the enzyme from S. cerevisiae and found it to have a mandatory requirement for bicarbonate in addition to the cofactors previously documented (31).

We further demonstrated the degradation of urea to be accomplished in a multi-step reaction catalysed by two distinct activities (33, 34): urea carboxylase which catalysed the reaction,

$$\text{UREA} + \text{ATP} + HCO_3^- \xrightarrow{Mg^{++}\ K^{+}} \text{ALLOPHANATE} + \text{ADP} + P_i$$

and allophanate hydrolase which catalyses the reaction,

$$\text{ALLOPHANATE} \longrightarrow 2\ \text{"}CO_2\text{"} + 2\ NH_3$$

The Genetics of Allantoin Degradation

We have isolated several thousand mutants which are unable to degrade one or more of the allantoin pathway intermediates. Loci containing many of the mutations harbored in these strains are situated in three clusters: one cluster on the right arm of chromosome IX contains the structural genes for allantoinase (dal1), the allantoin permease (dal4) and allantoicase (dal2)in that order (4, 16); a second cluster on the right arm of chromosome II contains the complementation groups for urea carboxylase (dur1) and allophanate hydrolase (dur2) (5); and a third cluster linked to the centromere of chromosome VIII, contains the gene(s) for the active transport of urea (dur3) and its facilitated diffusion (dur4)(ref. 24 and Turoscy et al., in preparation). The remaining genes associated with allantoin degradation are unlinked to one another or to these clusters.

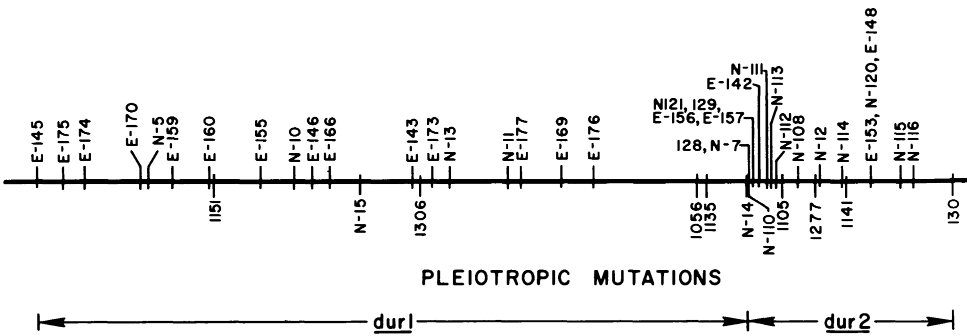

Figure 2. A fine structure recombinational map of the combined _durl_ and _dur2_ loci on the right arm of Chromosome II with the approximate locations of pleiotropic mutations indicated by the mutation designations appearing below the bold line. The extents of the _durl_ and _dur2_ domains are also indicated.

Early studies focused on the biochemical and genetic organization of the elements that participate in the degradation of urea. Mapping of mutations which resulted in the loss of urea carboxylase and allophanate hydrolase produced the fine structure map shown in Fig. 2 (5). Mutations that result in the loss of urea carboxylase activity were clustered at one end of the map, while those which result in the loss of allophanate hydrolase activity were clustered at the other end. Since _durl_ mutations complemented _dur2_ mutations, it was originally thought that two functional entities were involved in urea degradation (33). The two enzyme activities could, however, be extensively co-purified (30, 33) suggesting that urea carboxylase and allophanate hydrolase were either activities of a multienzyme complex or a multifunctional protein. The isolation of a third class of mutants gave further support to the idea that the two enzymatic functions were highly interactive. These pleiotropic mutants lacked both enzyme activities needed to degrade urea and the mutations they contained were unable to complement either _durl_ or _dur2_ mutations (5). Fine structure mapping demonstrated that the pleiotropic mutations were scattered throughout both _dur_ loci. When the urea-degrading protein was purified to homogeneity (Figure 3 and Sumrada and Cooper in preparation) using affinity chromatographic methods which avoided proteolytic clipping, a single polypeptide (apparent molecular weight = 204 kdaltons) possessing both enzyme activities was obtained. Amino acid composition studies of this protein suggested that it was composed of about 1887 amino acids and was quite acidic. From these observations Sumrada and Cooper concluded that urea carboxylase and allophanate hydrolase were component activities of a multifunctional protein and that the _durl_ and _dur2_ loci were two domains of a single monocistronic gene (_durl_, 2). This interpretation is being tested further using chimeric plasmids containing both the _durl_ and _dur2_ sequences (Genbauffe and Cooper,

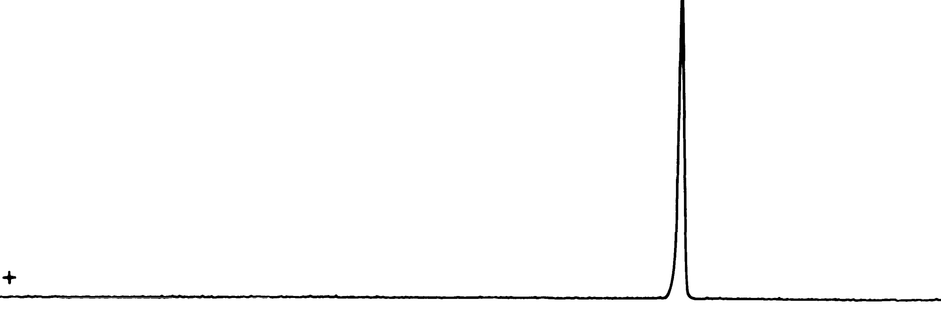

Figure 3. A densitometer tracing of an SDS gel on which purified urea amidolyase (urea carboxylase + allophanate hydrolase) was subjected to electrophoresis. The direction of migration was from (-) to (+).

in preparation). These probes permit the sizing of dur1,2 transcripts. If the dur1 and dur2 loci represent two domains of a single large gene, a single transcript of about 5.7 kb is expected. The sizing data obtained thus far are congruent with these expectations.

Induction of the Allantoin Degrading Enzymes

All four enzymes of the allantoin pathway and the urea active transport system are inducible; allophanate, the last intermediate of the pathway functions as the native inducer (6, 35). The allantoin transport system is inducible, but with allantoin itself functioning as inducer; hydantoin and hydantoin acetate serve as non-metabolizable inducers (25). The allantoate active transport (dal5) and urea facilitated diffusion (dur4) systems both appear to be produced constitutively (23, 28).

The involvement of allophanate in the expression of five distinct genes (dal1, dal2, dal3, dur1,2 & dur3) suggested that production of their cognate products might be regulated by common elements. This suggestion has been supported recently by the isolation of three new classes of mutants. Strains possessing lesions in the dal80 locus (formerly dur5) (3; Chisholm and Cooper in preparation) produce the four allantoin degrading enzymes and the urea active transport system at high, constitutive levels. Figure 4 depicts the amount of urea amidolyase (the 204 kdalton species) found in wild-type and dal80 mutant strains grown in the presence and absence of inducer. The gross, constitutive, overproduction of enzyme may be easily seen by comparing the amounts of protein found in lanes designated W. T. and dal80/dal80. Comparable constitutive levels were also observed in dur1, dal80 double mutants (Figure 5). This and the observation that arginase activity remains at its uninduced, basal level in Dal80 minus strains

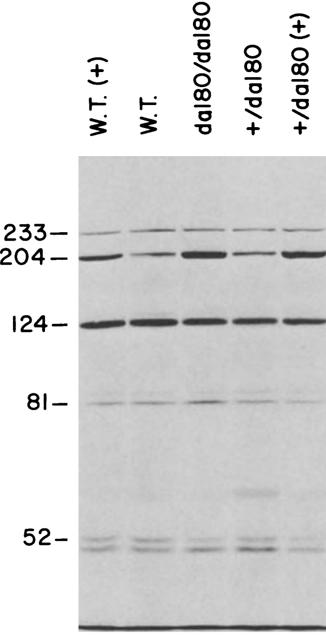

Figure 4. An audoradiograph of immunoprecipitated extracts separated by means of SDS gel electrophoresis. These extracts were derived from wild-type cells (W.T.), homozygous dal80/dal80 and heterozygous +/dal80 diploid strains grown in the presence (+) or absence or inducer. The 204 kdalton species is urea amidolyase, the protein whose concentration was monitored.

eliminates internal induction as the basis for constitutive enzyme synthesis. Mutations in dal80 are recessive to wild-type alleles (Figures 4 and 5). The dal80 locus has been located on the yeast genome and is not linked to any of the structural genes whose expression is regulated.

While attempting to identify strains unable to use allantoin, allantoate or urea as nitrogen source, we discovered two new classes of putative regulatory mutants (Turoscy *et al.*, in preparation). Strains from both classes grew poorly when provided with any of the allantoin related metabolites as sole nitrogen source, but used other nitrogen sources normally (Table I). Mutations found in these two sets of strains are recessive to wild-type alleles and complement mutations in all known loci associated with the allantoin pathway. Representative mutations from each class also complement one another. The locus containing the most thoroughly studied mutation has been designated dal81 and is not tightly linked to any of the allantoin pathway structural genes or to the dal80 locus. As shown in Figure 6, the levels of allantoin pathway enzymes observed in Dal81 minus strains are the same whether or not inducer is present

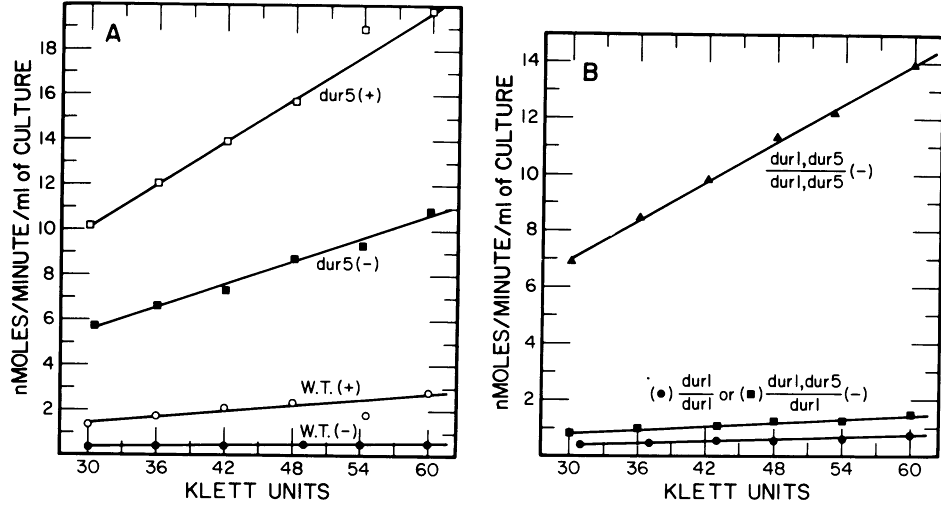

Figure 5. Differential rate of allophanate hydrolase synthesis in wild-type (W.T.), dal80 (indicated in this figure by the formerly used designation, dur5), and durl, dal80 double mutants grown on proline (-) or proline plus oxaluric acid (+). All of the strains used here were diploid and the pertinent genotypes are indicated on each curve in the figure. Note that comparison of the curves derived from strains with the genotypes (durl,+/durl, dur5) and durl, dur5/durl, dur5) permits a quantitative assessment of the dominance of the wild-type allele over the mutant dur5 or dal80 allele.

TABLE I
Growth of Wild-Type and dal81 Mutant Strains of Saccaromyces cerevisiae on Various Nitrogen Sources

Nitrogen Source	Doubling Time (Minutes) Wild-Type	*dal81*
Ammonia	130	135
Allantoin	155	290
Allantoate	215	350
Ureidoglycollate	245	430
Urea	175	365
Arginine	165	170
Proline	340	305
Asparagine	132	145
Citrulline	195	235
Proline + U.S.A.	N.G.	N.G.

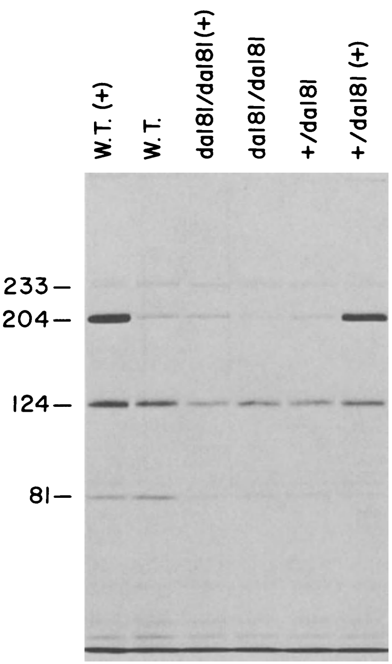

Figure 6. An audoradiograph of immunoprecipitated extracts separated by means of SDS gel electrophoresis. These extracts were derived from wild-type (W.T.), homozygous dal81/dal81 and heterozygous +/dal81 diploid strains grown in the presence (+) or absence of inducer. The 204 kdalton species is urea amidolyase, the protein whose concentration was being measured.

in the growth medium. However, the observed enzyme levels increased modestly when the mutants are grown on poor nitrogen sources. From these observations it was concluded that strains harboring mutations in the dal81 locus possessed an apparent defect in enzyme induction; enzyme derepression, however, appeared normal.

A second group of strains were found to harbor mutations that are not allelic to those in the dal81 locus, but generate the same phenotype. The locus containing these mutations has been designated dal82.

If, as we suspect, the allantoin degradative system is regulated by multiple regulatory elements, it is important to understand which of the elements interact with the pathway structural genes, the inducer and how they interact with one another. Our first approach to this question was to determine the epistasis relationship between dal80 and dal81 mutations. This was done by constructing a dal80, double mutant and ascertaining its phenotype. We found mutations

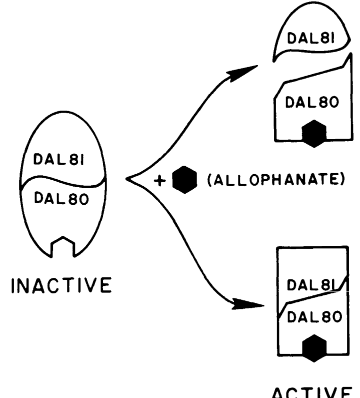

Figure 7. A working model to account for the genetic results obtained with mutants harboring lesions in the dal80 and dal81 loci. In this model both elements are synthesized constitutively. In the absence of inducer (allophanate) a complex forms between the DAL80 and DAL81 gene products. As a result of complex formation the DAL81 gene product is inactivated. In the absence of an active DAL81 gene product the five structural genes are incapable of expression. When allophanate is added, the complex is either dissociated (top figure) or its form is altered (bottom figure) thereby activating the DAL81 gene product. The active product may then associate with target sites adjacent to the allantoin system structural genes. This association results in expression. Conceptually similar models have been suggested by both Oshima and Hopper for the regulation of the GAL system of S. cerevisiae.

in the dal81 locus to be largely epistatic to those in the dal80 locus. In other words, the dal80, dal81 double mutant is uninducible but possesses a somewhat higher basal level than observed in the dal81 mutant. At 37°C the basal level observed in the double mutant drops to that seen in the dal81 mutant.

The model shown in Figure 7, is one way of visualizing our observations. According to the model, the dal81 gene product must be active for expression of the five allantoin pathway structural genes. The dal80 gene product can inactivate the positive-acting, dal81 product by forming a complex with it. Such interaction may be prevented naturally by the native inducer, allophanate, or artificially by mutationally rendering the dal80 gene product defective. How much, if any of this model is correct is not clear, but many of the questions it raises are. It hypothesizes that the regulated, structural genes are transcriptionally controlled and possess common target sequences which may perhaps be identified biochemically by sequencing the 3' regions of these genes or genetically by isolating mutants harboring lesions in the target region. Both questions have been addressed already.

The specific biochemical events associated with induction were extensively studied using allophanate hydrolase as a representative marker (2a,13-15). From these studies we concluded that allophanate hydrolase is induced by the tandem operation of two temporally separated processes; induction of the other pathway enzymes is believed to occur in a similar manner. First is specific accumulation of an allophanate hydrolase specific synthetic capacity; this accumulation requires inducer and RNA synthesis, but does not require protein synthesis. Second, this synthetic capacity is expressed; expression requires protein synthesis, but does not require RNA synthesis or the presence of inducer. The requirements of the two processes are those expected for the synthesis and translation of a specific mRNA.

A second result of the induction studies was identification of the functional half life of allophanate hydrolase specific synthetic capacity (7,14,15). Studies of collective mRNA metabolism in S. cerevisiae yielded synthetic capacity (presumed to be mRNA) half lives of 15 to 20 minutes (11,12,26). In contrast, we observed a value of 2.5 to 3.0 minutes for allophanate hydrolase specific synthetic capacity. The observed short synthetic capacity half life was extended to several other specific systems (2,7,17) and led to the conclusion that there is a wide spectrum of synthetic capacity stabilities ranging from those, like allophanate hydrolase, that are labile to those like the glycolytic enzymes that are highly stable. Failure to detect the labile species in experiments measuring global turnover derived from a lack of resolution in the techniques used as shown by Bossinger and Cooper (2). The biochemical basis for these differeing synthetic capacity stabilities remains obscure.

Although the above observations provide a good first approximation of the induction process and raise a number of provocative questions, it must be kept in mind that the conclusions were based on measurements of enzyme activity and hence were indirect. This deficiency is now being corrected. Recently, we have isolated and characterized chimeric plasmids each containing one of the allantoin pathway structural genes. As shown in Figure 8, radioactive polyA$^+$ RNA derived from cells provided with inducer hybridizes to DNA fragments known to contain portions of the dal2 gene. PolyA$^+$ RNA derived from cells grown in the absence of inducer did not hybridize to these DNA fragments. Though still incomplete, these data are consistent with our earlier hypothesis that induction and repression of the allantoin pathway structural genes derives from transcriptional control of gene expression. We have also tested polyA$^+$ RNA preparations derived from dal80 and dal81 mutant strains to determine whether or not the products of these genes similarly exert their effects on transcription.

There are two means of identifying the structural gene control regions hypothesized in Figure 7. Since all five genes responding

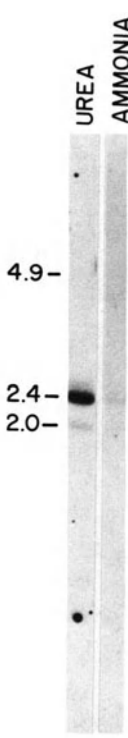

Figure 8. Hybridization of polyA+ RNA derived from wild-type S. cerevisiae grown with urea or ammonia as sole nitrogen source. The former nitrogen source will generate inducer of the allantoin degradative enzymes, whereas these enzymes are produced at much lower levels when the latter nitrogen source is provided. The source of DNA used in these southern blots was pTC-7 cut with SacI, HindIII, and SalI. This treatment yields DNA fragments with sizes of 4.9, 2.4, and 2.0 kb respectively. The positions for each fragment are indicated in the figure. Only the 2.6 and the 2.0 kb fragments contain dal gene sequences.

to allophanate induction also respond to each of the putative control elements, it is reasonable to suggest that the five genes all possess a homologous sequence recognized by the element(s). This homologous sequence may be apparent when the 3' terminii of all five structural genes are sequenced; this is in progress using the plasmids we have already characterized. On the other hand, the structure of the control element target may be more subtle. Here a tag is needed. If regulation of the genes is negative, a cis-dominant

TABLE II

Arginase and Ornithine Transaminase Activities in Wild Type and Mutant Strains of Saccharomyces cerevisiae

Strain Genotype	Arginase		Ornithine Transaminase	
	NH_4	NH_4+ arginine	NH_4	NH_4 + arginine
	Moles per hour per mg protein at 30°C			
Wild-type	6	18	0.03	0.24
car80	32	59	1.5	2.5
car10⁻	210	195	0.06	0.26
car20⁻	9	---	3.2	3.4
arg81,82	3	3	0.026	0.032

Taken from Wiame (37).

point mutation might identify the needed sequences. If regulation is positive, the situation may be less straight forward. At any rate, we and others (2a,9) have isolated a class of mutants which produce urea amidolyase at high, constitutive levels in the absence of inducer. The mutations responsible for constitutivity are tightly linked to the structural gene affected. The mutant phenotype is only seen, however, in a, α, homozygous a/a or α/α cells. It is notably missing in homozygous a/α diploids. Work from several laboratories (38) and described elsewhere in this volume by E.T. Young has shown that a Ty element has been inserted into the putative regulatory region of the gene whose expression has been altered. The presence of the Ty element results in the aberrant expression pattern observed. This also appears to be the case for the dur1,2 constitutives as well. Sequencing across the interfaces of these inserted sequences may provide a crude indication of where to look for the target region.

ARGININE DEGRADATION

Middlehoven (18) was first to study the pathway of arginine breakdown in S. cerevisiae. He identified the products of the arginase reaction to be ornithine and urea as shown in Figure 1. Thereafter ornithine transaminase mediates transamination of the δ-amino group of ornithine to yield glutamate semialdehyde. Mutations possessing defective forms of arginase (car1) and ornithine transaminase (car2) have been isolated by several laboratories (35, 37). For the sake of simplicity, arginine catabolism will be viewed as two groups

of reactions: the conversion of arginine to glutamate semialdehyde and urea and the degradation of urea to ammonia and carbon dioxide; the latter reactions have been discussed already.

Induction of the Arginine Degrading Enzymes

The enzymes, arginase and ornithine transaminase were both reported to be inducible (18); arginine was identified as the native inducer. Homoarginine was subsequently identified by Whitney and Magasanik (36) as a gratuitous inducer. Six classes of mutants that possess defects in arginase induction have been isolated (Table II). The first class of mutations are in the car80 locus (formerly cargR) and are recessive to the wild-type alleles. These mutations result in constitutive production of both arginase and ornithine transaminase (37). While the car80 locus has not yet been located on the yeast genome, it is not linked to either of the structural genes whose expression its product modulates. In formal terms, these mutations generate a phenotype similar to those in the dal80 locus. The next two classes of mutations (designated car10$^-$ and car20$^-$) also result in constitutivity. However, here constitutivity is restricted to the expression of only one enzyme. Also the mutations are cis-dominant and each is linked to the gene whose expression is altered. That is to say, a car10$^-$ mutation is tightly linked to the car1 locus and results in constitutive synthesis of only arginase; ornithine transaminase activity remains unaffected. Similarly, car20$^-$ mutations are linked to the car2 gene and result in constitutive synthesis of only ornithine transaminase. It is conceivable that these mutations are located in a regulatory target site adjacent to the gene whose expression is being modulated.

The remaining three mutant classes all possess the same recessive phenotype and are designated arg80, arg81 and arg82 (1). The defective loci have been located on the right arm of chromosome XIII, Fragment 8 and the right arm of chromosome IV respectively (19). The phenotype of arg80, 81 and 82 mutants has two major characteristics: (1) the mutants produce the arginine biosynthetic enzymes constitutively and (2) are unable to use arginine or ornithine as sole nitrogen source. Wiame and his colleagues have shown that mutants with defects in these three loci lack arginase and ornithine transaminase activities following addition of inducer, but appear to transport arginine normally into the cell.

There is an easily recognizable similarity between phenotypes generated by mutations in the arg80-82 loci and those in dal81 and dal82 loci. Their modes of action, however, may be quite different. It will be recalled that in the case of allantoin degradation that mutations generating uninducibility were epistatic to those yielding constitutivity. Here, mutations in the car80 locus, which generate constitutivity, are epistatic to those in the arg80-82 loci which result in uninducibility (37). A second striking difference between

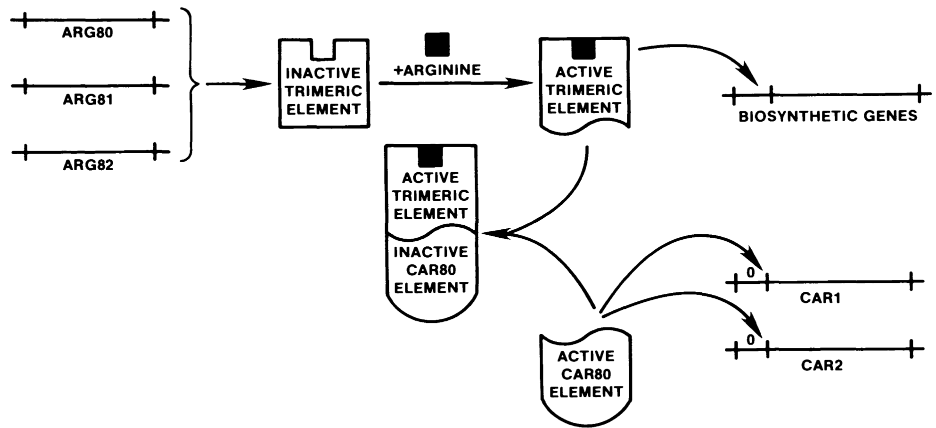

Figure 9. A working model to account for the genetic results obtained with mutants harboring lesions in the car80, car10⁻, car20⁻, arg80, arg81 and arg82 loci. In this model the ARG80, ARG81 and ARG82 gene products encode a trimeric regulatory element. In the absence of arginine, this element is unable to block target sites adjacent to the structural genes of the arginine biosynthetic system; hence the biosynthetic genes are expressed. The element is also unable to associate with the CAR80 gene product, which is then available to inactivate expression of the CAR1 and CAR2 genes by binding to target sites adjacent to them. When arginine is provided the trimeric control element is able to prevent expression of the biosynthetic genes and associate with the CAR80 gene product thus inactivating it. This inactivation results in the expression of the CAR1 and CAR2 genes.

the two systems is that defective arg80-82 gene products affect the levels of both anabolic and catabolic enzymes. These characteristics prompted Wiame to conclude that the arg80-82 loci encode a trimeric "ambivalent repressor" made up of three nonidentical subunits (8, 37). The model proposed to account for the genetic observations is shown in Figure 9. It is similar in many ways to the one shown in Figure 8; the roles of the dal80 and car80 gene products, however, would be reversed.

While the existence of a trimeric "ambivalent repressor" is certainly plausible, it is equally possible that the three gene products act independently. This action may be of a purely regulatory nature or alternatively may involve metabolic interconversions as well. At present there is insufficient data to distinguish between the possibilities. However, they generate unique and testable predictions. An important implication of the model shown in Figure 9 and the high degree of interaction hypothesized for the arg80-82 gene products is the isolation of mutations in any two of

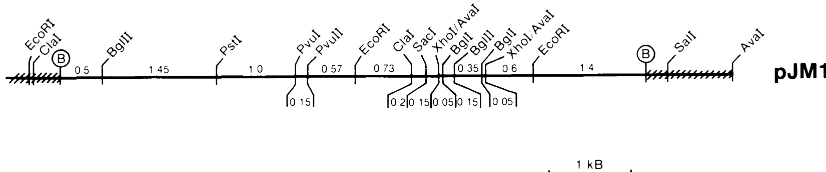

Figure 10. Restriction map of a plasmid carrying one of the ARG80-82 genes. This plasmid is able to support high frequency transformation of a strain that is unable to grow when provided with either arginine or ornithine as sole nitrogen source. This is the phenotype of mutants carrying lesions in the arg80-82 loci. Two other plasmids were also isolated that share sequences in common with the one shown in the figure. Both are also able to support high frequency transformation of the recipient mutant strain. All three plasmids are being analysed to determine which portions are needed for positive complementation.

the arg80, arg81 or arg82 loci which suppress, in an allele specific way, the phenotype of a mutation in the third locus. Such mutants have not yet been reported though many mutations have been isolated which reverse the effects of mutations in the arg80-82 loci (this is how the car10⁻ and car20⁻ mutations were isolated). The model also predicts the existence of target sites near the arginine anabolic and catabolic genes which interact with the putative aggregate product of the arg80-82 genes. If these target sites are non-identical for the catabolic and anabolic genes, it should be possible to isolate arg80-82 mutants which: (1) produce the anabolic enzymes constitutively and are able to use argine or ornithine as sole nitrogen source and (2) retain normal control of the anabolic enzymes and are unable to use arginine or ornithine as sole nitrogen source. Finally, it would be interesting to know whether or not expression of the arg80-82 genes is regulated. Here, some limited progress has been made with the isolation of chimeric plasmids containing one of the arg80-82 genes (Figure 10 and Cooper and McKelvey, in preparation). By fusing the regulatory region of the arg80-82 genes to the E. coli lacZ gene, we should be able to follow the expression of these genes with ease by measuring the amounts of β-galactosidase activity produced under various physiological circumstances.

The model also predicts transcriptional control of car1 and car2 gene expression. Bossinger and Cooper (2) demonstrated, in a set of experiments analogous to those described for the allantoin degrading enzyme, allophanate hydrolase, that induction and repression of arginase are probably mediated at the level of transcription. The arginase specific synthetic capacity was also shown to

possess a short (four to five minutes) half life leading to the conclusion that carl specific mRNA is quite labile. These conclusions have now been verified using recombinant plasmids containing the carl gene as a hybridization probe for the assay of arginase specific mRNA (Sumrada and Cooper, in preparation). Data very similar to that shown in Figure 8 has been obtained. We are also testing polyA$^+$ RNA derived from strains carrying lesions in the arg80-82 and car80 loci to ascertain whether or not the product of these loci exert their effects at transcription.

WHAT OF THE FUTURE?

The preliminary characterization data presented above suggests that control of carl and durl,2 gene expression is different even though both gene products are components of the same metabolic pathway. If the observations are interpreted at face value, carl seems to be subject to negative control, while the durl,2 gene is probably positively regulated. The differences seen for control of genes with closely related functions speaks in superlative terms of the broad spectrum of control mechanisms that might be observed as more diverse systems are studied. However, it would be a mistake to conclude that we understand either of the systems discussed. We have collected just enough information to begin formulating reasonable questions. We have presented models, but only as vehicles to aid in these formulations and in the design of future experiments. Delineation of the precise biochemical events that are lost in the mutants that have been isolated by others and ourselves is the next objective.

Returning to the beginning, if the genetics of procaryotic and eucaryotic control elements are compared a striking difference is seen. For most procaryotic systems only one or two elements participate in the control of gene expression. If, on the other hand, the initial observations described above can be substantiated by future experiments, eucaryotic cells seem to possess a more complex regulatory system involving significantly greater numbers of elements. This is further emphasized by the fact that we have confined our remarks to the induction of nitrogen catabolic systems. Expression of the car, dal and dur genes is also subject to nitrogen repression when cells are grown in the presence of readily used nitrogen sources. The elements that mediate this repression, however, remain obscure. Also unclear is the role played by chromatin structure in the control of eucaryotic gene expression and whether or not this more complex template structure generates the need for the additional control elements. What is clear is our need for answers to these questions and others like them if we are to exploit eucaryotic genes for commercial purposes.

ACKNOWLEDGEMENT

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STRATEGIES FOR CLONING IN BACILLUS SUBTILIS

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INTRODUCTION

In the past few years we have been involved in attempts to generate a versatile cloning system for Bacillus subtilis. We have had many failures and a few successes. It is our hope that the successful strategies will be applicable to a wide variety of microorganisms.

We began with a large advantage in B. subtilis as twenty years worth of genetics allowed us to construct a fairly detailed chromosomal map of this organism. These classical genetic studies have been carried out mainly with two systems of genetic exchange, DNA mediated transformation and PBS-1 mediated transduction. DNA mediated transformation is very useful for the transfer of very small pieces of DNA, from 1 kilobase or less to at least 30 kilobases. PBS-1 mediated transduction, on the other hand, transfers somewhere in the order of 300-400 kilobase pairs of DNA and thus allows a large segment of the chromosome to be cotransduced. The result of using these two systems has been the construction of the detailed chromosomal map shown in Figure 1. This map consists of at least 200 different genes of B. subtilis representing both auxotrophic mutations and developmental mutations in sporulation and germination (9). In addition, considerable work has been carried out on various temperate bacteriophage and studies of suppressors and other genetic tools have been accomplished. B. subtilis therefore emerges as the most carefully and well studied of the gram-positive organisms and allows it to serve as paradigm for this large group of microorganisms.

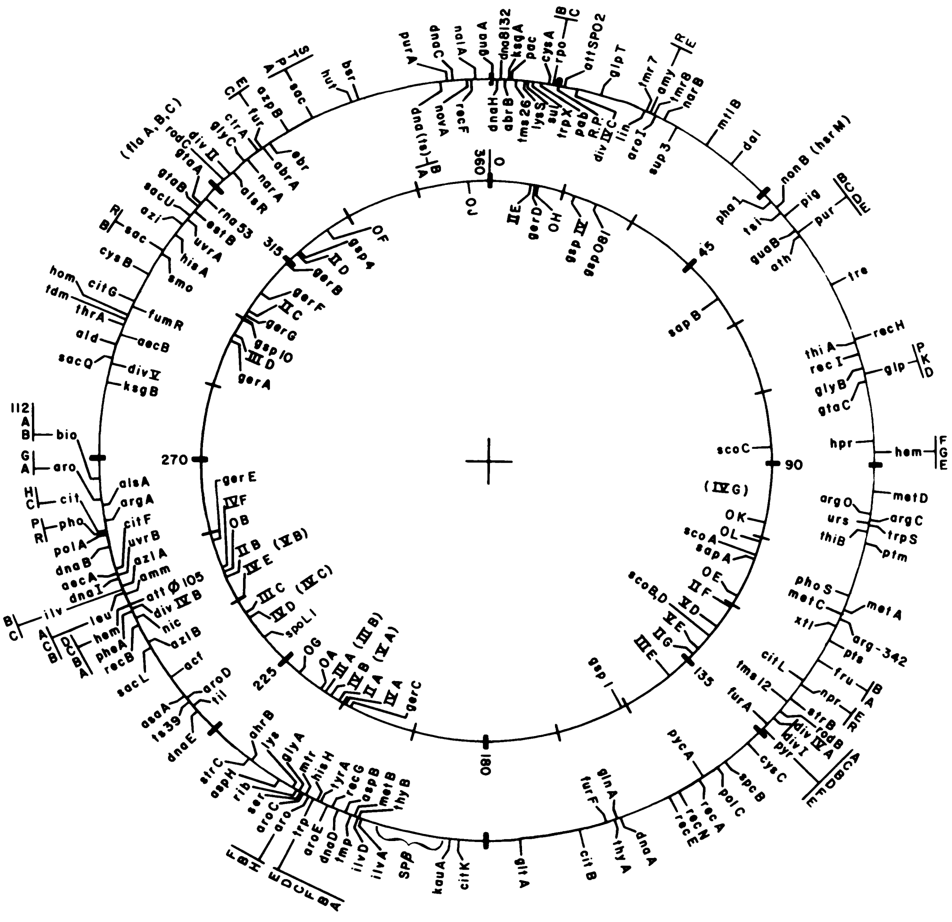
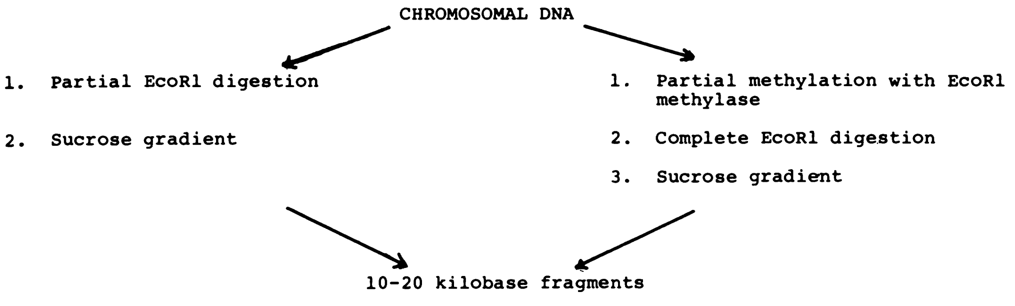


Figure 1. Genetic map of Bacillus subtilis chromosome.

CLONING LARGE FRAGMENTS OF THE BACILLUS SUBTILIS CHROMOSOME

It was desirable to isolate in a cloned form fairly large fragments of the B. subtilis chromosome in order that molecular studies of specific genes could be undertaken. We considered and rejected quite early in our adventures in cloning to attempt to generate a large fragment cloning vector like that of bacteriophage λ. After reviewing what was known of the restriction maps and regions of nonessential genes in the known temperate bacteriophage it became clear that to generate a system even approaching the versatility of λ was simply out of the question. Thus we have spent considerable time using the λ Charon bacteriophage for cloning of B. subtilis genes (5). Cloning has been carried out using EcoRI fragments of the B. subtilis chromosome in Charon 4 or more recently



ligate to purified arms of Charon 4

package *in vitro*

plaques

transformation in *B. subtilis*

Figure 2. Scheme for the generation of Charon 4 libraries.

because of the loss of certain regions by EcoRI digestion we have turned to cloning of Sau3A fragments of *B. subtilis* DNA in the BamHI vector Charon 30 (Figure 2).

Two different ways of obtaining EcoRI fragments for insertion into Charon 4 were used. Partial EcoRI digestion followed by sizing on a sucrose gradient was used to generate large fragments or a partial methylation with EcoRI methylase of the chromosomal DNA followed by a complete EcoRI digestion was also used. Large fragments were ligated to the purified arms of Charon 4, packaged *in vitro* and plaques used to transform *B. subtilis*. In our analysis of libraries prepared by both of these methods it was found that libraries prepared by partial EcoRI digestion of chromosomal DNA resulted in a highly nonrandom selection of sequences in the final library. Many genetic markers were found in fact never to reside in the 10-20 kilobase region upon partial EcoRI digestion. That is they moved from the chromosomal DNA area to pieces smaller than 10 kb. In order to attempt to overcome this problem we used a partial methylation scheme where the methylation of the DNA with EcoRI digestion. This results in a much more random library although several markers cannot be found in this library either. The results of these efforts have allowed us to isolate a wide variety of λ bacteriophage containing various regions of the chromosome that include both auxotrophic and developmental markers and we estimate approximately 10% of the *B. subtilis* chromosome now exists as identified and isolated fragments in λ.

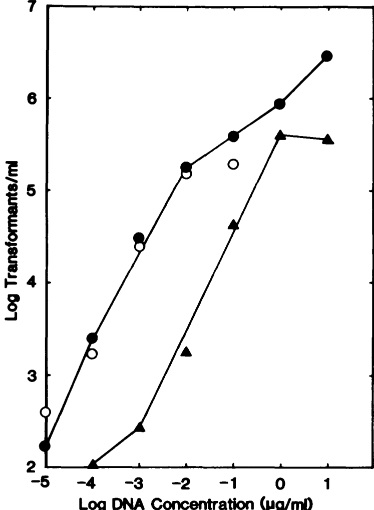


Figure 3. Number of the phe+ transformants at different DNA concentrations, using W168 DNA (▲) and λC4BsEF10 DNA (●) or λC4BsEF10 phage particles (●) as a donor and JH648 (trpC2, pheA1, spo0B136) as a recipient. For the lysate, 2 x 10^{10} plaque-forming units are assumed to contain 1 μg of DNA.

Transformation by DNA extracted from λ particles was found to be highly efficient (5). Figure 3 shows the number of transformants obtained as a function of DNA concentration for total chromosomal DNA and for DNA obtained from a λ phage that contains the phenylalanine marker. One thing that is noticeable is that the *pheA* λ is approximately 100-times more efficient on a DNA basis than total chromosomal DNA. In addition we were surprised to find that whole bacteriophage λ is capable of transforming *B. subtilis* without extraction of the DNA and also this transformation is insensitive to high concentrations of DNase. Somehow the λ is capable of entering the cell and effecting transformation. The high efficiency suggests that the flanking regions of phage DNA on either side of the insert have little if any effect on the transformation frequency of the insert DNA in these Charon phage. Also there appears to be very little effect of cycling the DNA through *E. coli* on the ultimate transforming ability in *B. subtilis*. In conclusion the cloning of *B. subtilis* DNA in the *E. coli* Charon vectors has proven to be highly useful for the isolation of a large variety of fragments of the *B. subtilis* chromosome. In addition the identification of the various fragments is quite easy because of the transformation system and there appears to be little interference with the DNA's genetic activity by being cloned in λ.

CONSTRUCTION OF BIFUNCTIONAL PLASMIDS

When we began to think about cloning in plasmids in B. subtilis there were only a few known plasmids of this genus and all these were cryptic. At this time it became evident that plasmids that replicate in Staphylococcus aureus can, in general, replicate in B. subtilis (3). This fact brought on a wealth of possibilities because a large number of antibiotic resistance plasmids have been found in clinical isolates of S. aureus and it appeared at that time simple to use these plasmids for cloning in B. subtilis. These expectations were not wholly realized. There turn out to be a number of yet unresolved problems in directly using S. aureus plasmids for cloning in B. subtilis. The only documentable successes using S. aureus plasmids for cloning in B. subtilis have been the use of these plasmids with heterologous DNA, that is DNA that does not recombine readily with the B. subtilis chromosome (6,10). Thus DNA from Bacillus licheniformis and Bacillus pumilus, for example, have been successfully cloned and maintained in B. subtilis strains. In the homologous situation it appears that no stable plasmids carrying homologeous DNA are obtained in cloning experiments that give successful results with heterologous DNA. This most likely results from the fact that even in our best recombination deficient strains of B. subtilis incoming recombinant plasmids probably are capable of recombination with the recipient chromosome and are lost.

Thus it became clear that in order to obtain small fragments of B. subtilis DNA cloned into plasmids we were going to have to use a heterologous system other than B. subtilis in order to obtain these plasmids. The only logical alternative heterologous system to accomplish this cloning was E. coli and the well characterized E. coli plasmids. Most of our subcloning of fragments from B. subtilis DNA cloned in Charon 4 has been in the E. coli plasmids pBR322 and pBR328 (1,11). The more of this we have done the clearer it becomes that a homologous plasmid system for cloning and engineering of chromosomal fragments is less desireable than an heterologous one given the problems that can occur with recombination between chromosomal DNA and plasmids carrying DNA homologous to it.

It has been known for several years now that one can construct chimeric plasmids between E. coli plasmids such as pB322 and the S. aureus plasmids that express various antibiotic resistances (4,7). One of these constructed several years ago by Ehrlich consisted of S. aureus plasmid pC194 and pBR322 (4). Figure 4 shows the restriction maps of these two parental plasmids and the primary antibiotic resistance determinants in each. pC194 is a small multicopy plasmid in both S. aureus and B. subtilis expressing chloramphenicol resistance via the chloramphenicol acetal transferase gene. There are three other open reading frames in this plasmid, some of which probably make products required for replication of the plasmid.

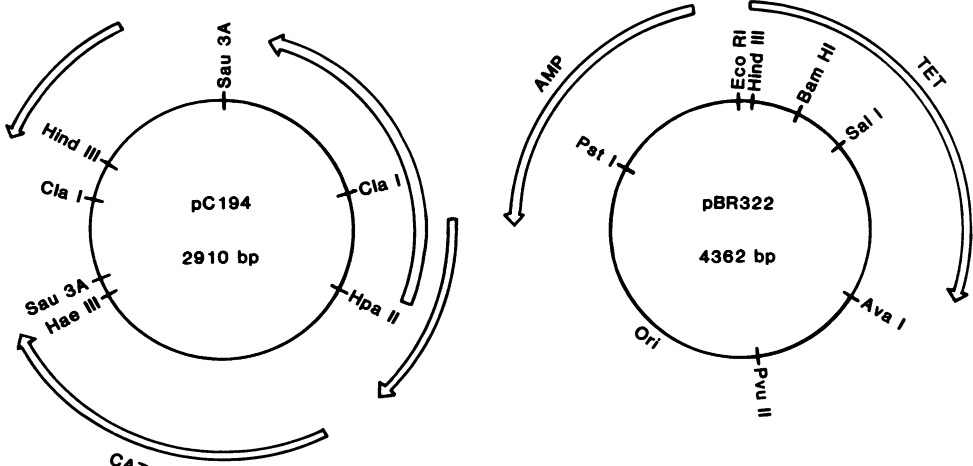


Figure 4. Restriction maps of plasmids pC194 and pBR322.

The entire nucleotide sequence of this plasmid has recently been determined by Dr. Weisbloom's laboratory at the University of Wisconsin. It was known from previous studies that the HpaII site was part of something required for replication, either the origin of replication or some protein required for replication of the plasmid (2). Joining plasmid pC194 with pBR322 through their single HindIII sites results in the plasmid pHV14. This plasmid was capable of replication in both E. coli and B. subtilis, expressed both chloramphenicol resistance and amplicillin resistance in E. coli and expressed chloramphenicol resistance in B. subtilis but not ampicillin resistance. This plasmid replicates well in E. coli and appears to replicate well in B. subtilis but has a partioning defect as it segregates at a fairly high rate from B. subtilis in the absence of chloramphenicol selection. We have recently constructed a more useful bifunctional replicon by leaving the ampicillin and tetracycline resistance genes intact such that a variety of restriction enzymes can be used to clone into either of these insertionally-inactivated resistance genes. In order to generate this more useful bifunctional replicon we have inserted random pieces of pC194 generated by cutting with DNAase I in the presence of manganese. These fragments have been inserted into the AvaI site of plasmid pBR322. This plasmid gives good growth in both E. coli and B. subtilis and all three resistances are expressed in E. coli but only chloramphenicol in B. subtilis. The plasmid should be quite useful for cloning fragments in a variety of gram-positive and gram-negative organisms and should allow one to shuttle the plasmid back and forth between a wide variety of evolutionary divergent microorganisms.

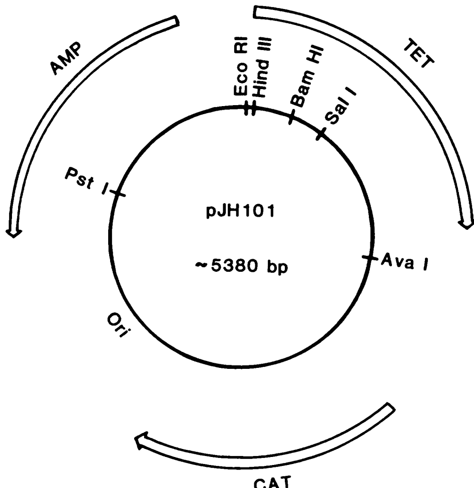


Figure 5. Restriction map of pJH101.

INSERTION ELEMENTS GENERATED *IN VITRO*

We have been able to generate some most interesting and useful plasmids using chimeras. We have cut the chloramphenicol resistance gene out of pC194 by cleavage at the *Sau*3A and *Hpa*II sites. This fragment of pC194 has been inserted into the *Pvu*II site of pBR322 by ligation after SI digestion. The resultant plasmid, pJN101, (Figure 5) is a chimera expressing chloramphenicol, ampicillin and tetracycline resistance in *E. coli* but only chloramphenicol resistance in *B. subtilis*. It does not contain an origin recognized by *B. subtilis* and therefore cannot replicate in B. subtilis. Plasmids of this type are extremely useful in genetic analyses in *B. subtilis* and have been used in two ways.

One way in which we have used similar plasmids is to map isolated fragments of *B. subtilis* DNA that cannot be studied by classical genetic means. One example of this is a fragment of DNA isolated by the Losick group at Harvard that contains genes that are activated during sporulation but contains no genetic markers. In order to determine where this fragment of DNA resided on the *B. subtilis* chromosome we simply inserted it into a plasmid with properties similar to pJH101. When a homologous fragment is inserted into such plasmids and the resulting plasmid is transformed into *B. subtilis* the only chloramphenicol resistant transformants one obtains are those in which the plasmid has become stably associated with the chromosome (8). Such a stable association occurs by Campbell-type recombination, Figure 6, between the incoming plasmid and the chromosome generating a tandem duplication that flanks the plasmid sequences this configuration for the plasmid is highly

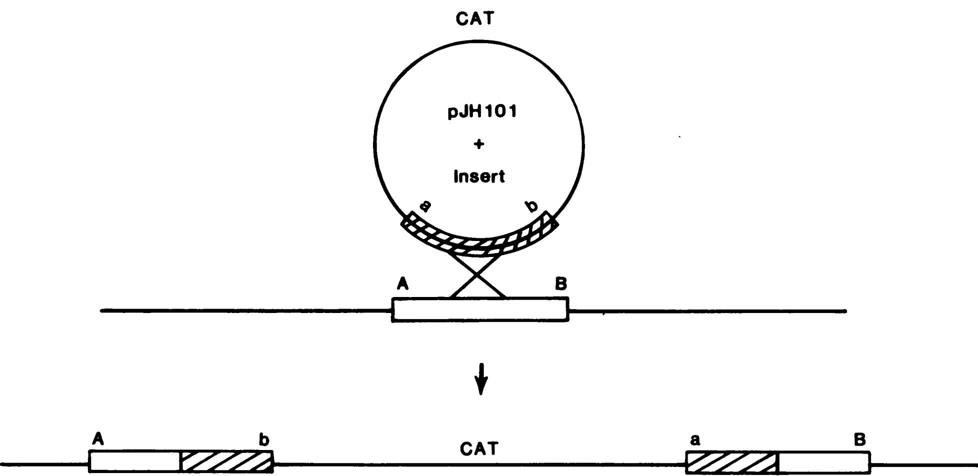


Figure 6. Scheme for the recombination of insert containing pJH101 into the *Bacillus subtilis* chromosome.

stable and the inserted plasmid can be mapped by both PBS-1 transduction and transformation and acts essentially as a point mutation in these crosses. Thus the cryptic piece of DNA can be readily mapped because of the chloramphenicol resistance marker. We have also used this technique to map the ribosomal RNA genes for which there was no easily selectible phenotype (12).

The second way in which these plasmids are useful is they can be used to generate insertion elements *in vitro*. Thus consider the case when a fragment of a gene is cloned that contains neither end of the gene or when a fragment is cloned that contains only an internal part of the operon. In these cases transformation of this plasmid into *B. subtilis* results in either insertional inactivation of the gene involved or the introduction of a large stretch of DNA separating operator distal genes from their regulatory regions. One can use these plasmids to generate mutations in what were up until now cryptic genes in a manner similar to transposons. Also they can be used to determine polarity, etc.

SINGLE COPY COMPLEMENTATION AND DOMINANCE SYSTEM

One of the chronic problems we've had in *Bacillus subtilis* genetics is the lack of a good system to study complementation and dominance. When it appeared that *S. aureus* plasmids were going to be useful for cloning in *B. subtilis* we had hoped that these plasmids might be useful in this regard. Since the *S. aureus* plasmids are all multicopy plasmids it was not clear how meaningful complementation and dominance studies are when one allele is in 20- to 50-fold

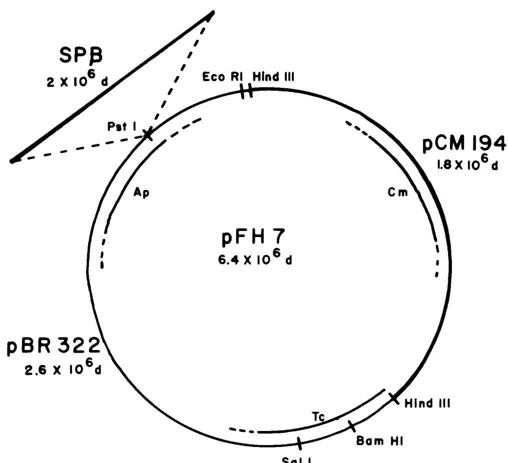


Figure 7. Restriction map of pFH7.

excess over the other complementing allele. Thus we set about to devise a single copy complementation and domominance system. How we did this was to take a fragment of the temperate bacteriophage SPβ that resides at about 180° on a *B. subtilis* chromosome and is indigenous to all *B. subtilis* strains. *Pst*I fragments of the bacteriophage were cloned into the ampicillin resistance gene of plasmid pHV14 consisting of a chimera between pBR322 and pC194. This resultant plasmid pFH7 (Figure 7) carries a strong region of homology to the *B. subtilis* chromosome and therefore integrates at very high frequency into the SPβ prophage yielding stable chlor-amphenicol resistant transformants. These lysogens have some interesting properties. Greater than 50% of the plaque forming units from induced culture are specialized transducing phage for chloramphenicol resistance. Thus the integration of the plasmid within SPβ does not interfere with its ability to induce and replicate. In the strains carrying this integrated plasmid there is no detectable free plasmid. The chloramphenicol resistance maps within the prophage genome by PBS-1 transduction. If one induces the bacteriophage and isolates the bacteriophage particles, one can use that DNA to transform *E. coli* and recover the original plasmid. The most interesting aspect of these plasmids is the fact that when pFH7 is integrated into SPβ it produces a region of homology to *E. coli* plasmids related to pBR322.

pBR322-related plasmids carrying *B. subtilis* DNA have two regions of homology with the *B. subtilis* chromosome, that of the insert DNA and of the plasmid itself. Transformation of these plasmids from *E. coli* into *B. subtilis* results in the integration of the plasmid either at the site of pFH7 in SPβ or in the region of homology to the insert. Plasmids integrated into pFH7 in SPβ

transduction. Although the present system is not the most advantageous from the standpoint of ease of manipulation, it shows the feasibility of using this approach to obtain facile complementation and dominance systems. Several more useful plasmids are in the process of being constructed.

In summary our strategy to generate molecular cloning vectors for Bacillus subtilis has been to make use of the E. coli vectors as much as possible. We believe these bifunctional replicons will be useful for cloning in a wide variety of both gram-negative and gram-positive microorganisms.

ACKNOWLEDGEMENTS

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DISCUSSION

Q. F. MACRINA: How do you envision the pFH7 plasmid being able to recircularize when introduced into *E. coli*?

A. HOCH: It has a tandem duplication of SPB on either side of it and probably comes out of a reversal of the way it went in. There might also be some free plasmid in *B. subtilis* that is transferred.

Q. SOMERVILLE: Can you make any general statements on the expressibility of *Bacillus subtilis*?

A. HOCH: I think there are both transcriptional and translational barriers that exist in *B. subtilis* to expression of some *E. coli* genes. The reverse case does not seem to be true.

GENETIC AND MOLECULAR STUDIES OF THE REGULATION OF ATYPICAL CITRATE UTILIZATION AND VARIABLE Vi ANTIGEN EXPRESSION IN ENTERIC BACTERIA

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INTRODUCTION

Genetic and biochemical studies of the metabolic and virulence properties of enterobacteria have been conducted, both for basic scientific and industrial reasons, for more than 40 years. Despite this long time span, only limited genetic characterization of enteric organisms other than *Escherichia coli* K-12 has been accomplished. Though several reasons can be offered for this lack of information, a major problem often encountered is the difficulty in genetically manipulating these other enteric organisms, e.g., *Salmonella typhimurium*, *Salmonella typhi*, *Citrobacter freundii*, wild-type *E. coli*, or *Shigella* species. In addition to facing DNA restriction barriers in many of these organisms, Hfr strains can not be easily constructed by classical procedures. Nevertheless, about 20 years ago my colleagues and I began intergeneric genetic transfer experiments with different enterobacteria to characterize their genetic constitution (2,3,8,13). Initially, classical genetic methods were used (i.e., F, F', and Hfr strains), but these techniques presented many problems and resultant events usually occurred at a low frequency. Despite the difficulties, these studies led to the initial descriptions of conjugative plasmids coding for lactose and sucrose utilization in *Salmonella* (7,18,26,28) and involved the first mapping of chromosomally located antigenic determinants of *Salmonella typhi*, the causative agent of typhoid fever (11,13,14). However, a constellation of new, rapid, molecular genetic techniques, developed over the past 10 or so years, has now made the genetic study of many of

these bacterial species more practicable. These techniques include rapid, simple procedures for plasmid DNA isolation, simplified DNA transformation procedures, the use of transposons and Mu phage to manipulate genes of interest, and recombinant DNA technology, to mention a few. Various combinations of these procedures have now been employed in characterizing several genetic systems which include λ bacteriophage, the lactose or tryptophan operons of *E. coli*, and the conjugal transfer (*tra*) operon of the F plasmid. In addition to these well-studied systems, many other bacterial chromosomal, viral, and plasmid traits have been analyzed similarly. We have been involved over the past few years in analyzing various unusual traits encoded by enterobacteria, some of which are plasmid-determined (4, 7,10,16,17,18,26,28). In this communication, we will focus on two such studies, one of a plasmid-determined property and the other of a chromosomally encoded function, and will describe the variety of different procedures employed in their genetic, biochemical and immunologic characterization. The first topic to be discussed is the atypical utilization of citrate by some natural isolates of *E. coli* and the second topic involves the initial steps in the study of a genetic "expression" switch mechanism that controls Vi antigen expression in *Citrobacter freundii*.

RESULTS AND DISCUSSION

I. Genetic and Biochemical Analyses of Atypical Citrate-Utilizing *E. coli*

Atypical citrate utilization of E. coli. Certain biochemical traits of bacteria are employed as key diagnostic aids in the clinical microbiology laboratory. Over the past 15 years, a variety of diagnostically important functions, such as hydrogen sulfide (17) or urease (9) production as well as the utilization of lactose (16, 26) or sucrose (18,28) have been found to be expressed atypically in some enterobacteria. Many of these functions have been demonstrated by genetic and molecular means to be plasmid-mediated. Awareness of the presence of such plasmids is necessary for the rapid and proper identification of atypical enterobacterial isolates, the misidentification of which might result in the administration of ineffective chemotherapeutic regimens to patients. Though *E. coli* are characteristically citrate-nonutilizing, Washington and Timm (27) reported in 1976 the isolation of several *E. coli* strains that were atypical only in their ability to utilize citrate. Subsequently, we decided to examine the nature of the citrate-utilizing ability in two such atypical *E. coli* isolates, which were isolated from diseased humans, that were obtained from the Center for Disease Control (CDC; Atlanta, Georgia). The results which follow represent a combined biochemical and genetic analysis of this metabolic function (4).

Diagnostic characterization of atypical E. coli strains. Citrate-utilizing E. coli strains, V414 and V517, were examined by routine diagnostic biochemical tests (19). As shown in Table I, these strains were typical E. coli except for the citrate-utilizing character. In addition, strain V414 was found to be resistant to tetracycline and chloramphenicol, whereas V517 expressed significant resistance to 3 different antibiotics (see Table I). Information obtained from the CDC and in our laboratory indicated that the citrate-utilizing ability of these strains was spontaneously lost at a relatively high frequency, a fact which suggested that plasmids might be involved in citrate-utilization.

Conjugal transfer of citrate-utilizing ability. Initially, attempts were made to transfer conjugally the citrate trait to appropriate recipient bacteria, as outlined in Table II. Auxotrophic derivatives of strains V414 or V517, made by nitrosoguanidine mutagenesis, were mated with a chromosomally-determined streptomycin resistant recipient E. coli strain 2340. Conjugal mating

TABLE I
Biotype and Antibiotic Resistance Patterns of Two Atypical Escherichia coli Strains

Strain Designation	Diagnostic Characteristics[a]	Antibiotic Resistance[b]
V414	Indole$^+$, Lactose$^+$, Melibiose$^+$ V-P$^-$, Glucose$^+$, Sucrose$^-$ gelatin$^-$, Mannitol$^+$, Inositol$^-$ urease$^-$, Rhamnose$^+$, Arabinose$^+$ H_2S^-, Sorbitol$^+$, Citrate$^+$ Ornithine decarboxylase$^-$ Lysine decarboxylase$^+$ Arginine dehydrolase$^-$ Tryptophan deaminase$^-$	Tetracycline Chloramphenicol
V517	Same as above	Amikacin Cephalosporin Kanamycin

[a]V-P = Voges-Proskauer; H_2S = hydrogen sulfide producing; + = either producing or utilizing whereas - = either non-producing or non-utilizing.

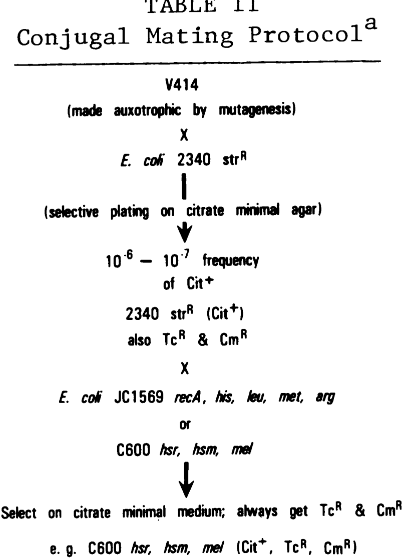
[b]Assessed by standard agar dilution method.

experiments were conducted at 37°C directly on selective minimal salts agar containing citrate as the sole carbon source. No conjugal transfer of the citrate character was observed from strain V517, even after repeated attempts utilizing several different auxotrophic derivatives of this strain. However, strain V414 did transfer the citrate character to strain 2340 at the relatively low frequency of 10^{-6} - 10^{-7} transconjugants per input donor cell. In addition, we noted that all of these citrate-utilizing transconjugants were also resistant to tetracycline (Tc^r) and chloramphenicol (Cm^r), the antibiotic resistance traits initially observed in strain V414. Subsequently, the citrate character was transferred from strain 2340 to other E. coli strains or to Salmonella typhi strains 643 at approximately the same low frequency. Selection for transconjugants on citrate minimal medium resulted in cotransfer of Tc^r and Cm^r. As one might expect, when conjugal transfer of Tc^r (or Cm^r) was the selected trait, both Cm^r (or Tc^r) and citrate-utilizing ability could be cotransferred with Tc^r (or Cm^r). Transfer of these three linked traits was not enhanced by conducting the conjugal matings at 25° or 32°C. These results indicate that the observed low frequency transfer is not due to a conjugal transfer system that is sensitive to temperatures above 30°C, such as has been reported from some plasmids that mediate citrate utilization (10,23). Thus, strain V414 harbors a conjugative plasmid that specifies resistance to tetracycline and chloramphenicol and that encodes certain functions which enable E. coli to utilize citrate.

Since no direct conjugal transfer of the citrate trait was detected from strain V517, an attempt was made to mobilize this character from V517 using the conjugally derepressed R64-11 plasmid; to date, these experiments have not been successful. However, purified V517 plasmid DNA has been used to transform competent E. coli recipients for citrate-utilizing ability. These results suggest that the atypical citrate-utilizing determinants of strain V517 are located on a nonconjugative plasmid.

Plasmid DNA analyses of citrate-utilizing E. coli strains. Following the conjugal transfer experiments, the parental V414 and V517 strains, the E. coli recipient strains, as well as the citrate-utilizing (Cit^+) transconjugants were examined for the presence of plasmid DNA. Cells grown in citrate minimal medium were gently lyzed with Triton X-100 detergent and plasmid DNA was separated from chromosomal DNA by centrifugation of cell lysates in cesium chloride-ethidium bromide gradients. A covalently-closed, circular plasmid DNA band was never observed in DNA preparations made from the plasmid-free recipient strains, but was always detected in the lysates of the parental V414 and V517 strains as well as in all Cit^+ transconjugant clones. Purified plasmid DNA from these strains was preliminarily examined by electrophoresis in 0.7% agarose gels, a technique allowing for the rapid separation of different molecular

TABLE II
Conjugal Mating Protocol[a]



[a]Conjugal mating experiments were conducted at 37°C on minimal agar containing citrate, with appropriate supplements. Relative transconjugal transfer frequency is expressed as the number of conjugants per input donor cell.

DNA species. The plasmid DNA obtained from strain V414 or any transconjugants that had received the Cit^+ trait from this strain, contained only a single plasmid species that appeared identical in all DNA preparations and was estimated to be 130 Mdal in size. Thus, this large plasmid presumably encodes resistance to tetracycline and chloramphenicol as well as genes involved in citrate-utilizing ability.

Although the Cit^+ ability was never transferred out of strain V517, this strain was found to harbor a minimum of nine distinct plasmid DNA species (19). From estimates obtained using standard plasmids of known molecular size, examined in side-by-side wells of the same slab gel, these V517 plasmid species ranged from approximately 1 to 40 megadaltons (Mdal) in size. Furthermore, Cit^+ *E. coli* transformants of V517 plasmid DNA were always found to carry the 36 Mdal plasmid species. Thus, the 36 Mdal plasmid of V517 encodes the atypical Cit^+ ability. More accurate molecular sizes for all of these plasmids were obtained by contour length measurement in the electron microscope. These sizes are given in Table III.

Cloning of the genes for citrate utilization. To prove that the 130 Mdal plasmid of strain V414, termed pWR60, actually encodes some determinants of citrate utilization and does not simply mobilize

TABLE III
Contour Length of Plasmids from *E. coli* Strains V414 and V517

Strain/(Plasmid)		no.[a]	Size of Megadaltons ± Standard Derivations
V414	(pWR60)	4	129.86 ± 5.75
V517	(pWR517-1)	5	35.84 ± 1.0
"	(pWR517-2)	18	4.82 ± 0.11
"	(pWR517-3)	25	3.74 ± 0.06
"	(pWR517-4)	29	3.60 ± 0.04
"	(pWR517-5)	29	3.39 ± 0.09
"	(pWR517-6)	19	2.63 ± 0.07
"	(pWR517-7)	34	2.03 ± 0.06
"	(pWR517-8)	76	1.79 ± 0.07
"	(pWR517-9)	61	1.36 ± 0.08

(a) Number of separate molecules measured from enlarged electron micrographs, as reported previously (19).

chromosomal Cit^+ genes, the putative plasmid-borne Cit^+ traits were cloned using recombinant DNA techniques. DNA of pBR325, the vector, and pWR60 were mixed and digested with the *Pstl* endonuclease. The resulting linear DNA fragments were ligated with polynucleotide ligase and used to transform competent *E. coli* cells (see Fig. 1). Several independent Cit^+ recombinant clones were obtained; the one shown here is termed pWR61. Plasmid DNA from each resulting Cit^+ recombinant was found by agarose gel electrophoresis to be 9.6 Mdal in size. In addition, when digested with *Pstl* and examined on agarose gels, each recombinant plasmid gave rise to 2 DNA fragments, one of 6 Mdal (i.e., 9 kilobase prs, (kb)) and the other of 3.6 Mdal (i.e., pBR325), as shown in Figure 2. Thus, the original 130 Mdal pWR60 plasmid does encode some determinants of citrate utilization. The Cit^+ gene(s) of pWR60 is located on a 9 kb *Pstl* fragment.

Biochemical characterization of citrate utilization. Normally, *E. coli* does not utilize citrate, apparently because citrate cannot be transported into the cell. Several metabolic studies were conducted to determine exactly what role citrate plays in the growth of these strains. Free iron is essential to the growth of all enterobacteria. Recent studies have shown that *E. coli* contain siderophores, termed enterochelin, that are involved in sequestering iron (5). Enterochelin-deficient *E. coli* mutants are not able to grow in medium containing low, free iron concentrations. However, the addition of citrate to the growth medium has been observed to enhance the growth of enterochelin-deficient *E. coli* strains,

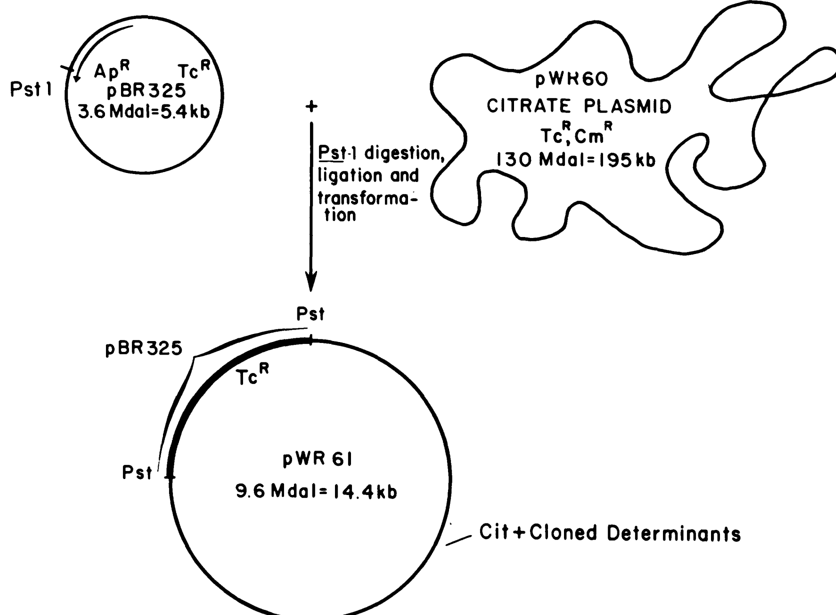


Figure 1. Cloning of the citrate utilization genes. pBR325 and pWR60 DNAs were purified from host bacterial strains by triton X-100 lysis followed by CsCl-EtBr gradient centrifugation and extensive dialysis, as described previously (19). 0.5 μg pBR325 DNA and 1.5 μg of pWR60 DNA were mixed, digested with Pstl endonuclease, and ligated in a 100 μl final volume with T4 polynucleotide ligase, using the buffers and conditions recommended by New England Biolabs. The location of the single Pstl site in pBR325 is shown. E. coli transformants for recombinant plasmids, initially selected on the basis of Tc^R and sensitivity to ampicillin, were later tested for Cit^+ ability.

presumably by serving as an iron-chelator (5). Thus, studies were conducted to determine if pWR60 somehow enhances the iron-sequestering ability of host bacterial strains. The plasmid pWR60 did not enhance the ability of either E. coli strain 2340 or the enterochelin-deficient mutant strain AN193 to grown under conditions of iron starvation (unpublished data). Therefore, although citrate can coincidentally enhance the growth of enterochelin-deficient E. coli strains, the plasmid pWR60 does not appear to encode an iron-sequestering system.

Next, metabolic studies were performed to see if radiolabelled citrate could be incorporated into these atypical citrate-utilizing E. coli cells, perhaps via a plasmid-mediated permease. To examine citrate uptake, cells were pregrown at 37°C in a casamino

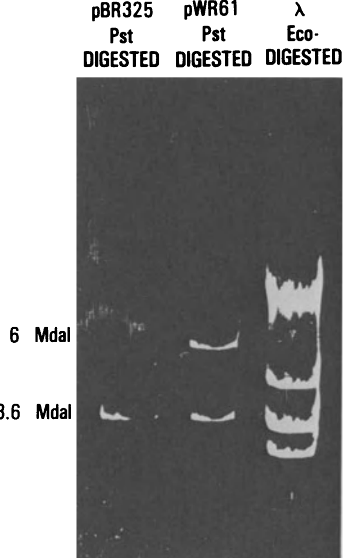


Figure 2. Agarose gel analysis of cloned citrate genes. Endonuclease-digested DNAs were electrophoresed for 2 hr at 150 V in a 0.7% agarose slab gel in Tris-borate buffer, DNA bands were stained with EtBr, and the resulting bands were photographed, as described previously (19). Pstl-digested pBR325 vector DNA is compared to the Pstl-digested Cit^+ plasmid, pWR61. Molecular sizes of the resulting DNA bands were estimated from the known EcoRl-digestion fragments of λ DNA.

acids-basal salts medium with or without citrate. These cells were washed, adjusted to a set cell density in fresh basal salts medium containing ^{14}C-1-5 labelled citrate (i.e., the external carbon atoms are radiolabelled), and incubated at 37°C. Samples were taken periodically for 120 minutes and examined for ^{14}C-label incorporation into TCA precipitable cellular material, radiolabelled carbon dioxide respiration, and also total protein to monitor cell growth. The results shown in Figure 3 were obtained with *E. coli* strain V414; the graph on the left represents the results obtained with cells pregrown in the absence of citrate, whereas the graph on the right represents the uptake studies on cells that were pregrown with citrate. Very little of the ^{14}C-label was incorporated into TCA-precipitable material over this time span, as represented by the bottom line in each graph. The majority of the utilized radiolabelled citrate carbon could be accounted for as expired CO_2, shown by lines marked with closed circles. In addition, the cells pregrown in citrate showed a 3-fold increase in the initial rate of ^{14}C-labelled carbon dioxide respired, over that seen with cells pregrown in the absence of citrate. Similar results have been

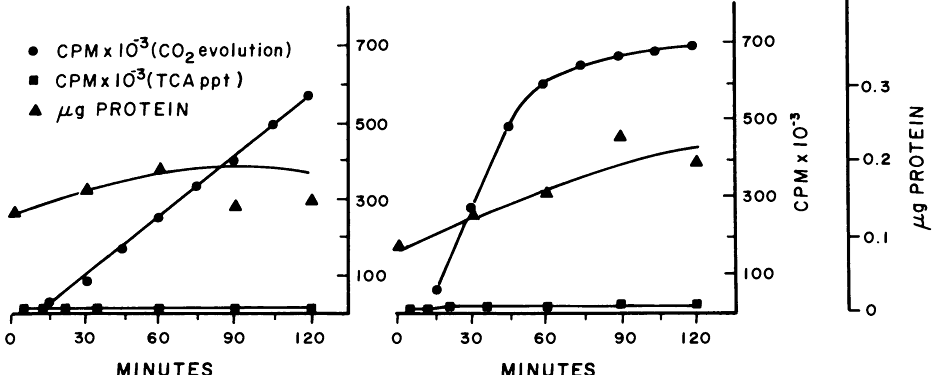


Figure 3. Studies on the uptake of ^{14}C-1-5-labelled citrate in E. coli V414. Cells were pregrown at 37°C in casamino acids-basal salts medium containing 20 mM lactate with (b) or without (a) 30 mM citrate. Pregrown cells were washed, resuspended at a set density in fresh basal salts medium containing 20 mM lactate and ^{14}C-1-5-labelled citrate. Incubation was continued at 37°C with aeration. Samples were taken regularly over a 120 min interval and examined for ^{14}C-incorporation into trichloracetic acid-precipitable cellular material, $^{14}CO_2$ respiration, and also total protein content (determined by Lowry method).

obtained with a citrate-utilizing transconjugant 2340 strain containing pWR60 and also with strain V517. As expected, the plasmid-free E. coli 2340 recipient strain neither incorporates label nor respires labelled CO_2 under similar conditions.

Other kinetic studies show that during the first 15 min of these uptake experiments, 90-95% of the radiolabelled carbons of the utilized citrate was evolved as labelled carbon dioxide. These results indicate that citrate is being metabolized extracellularly or perhaps at the cell surface, and that decarboxylation of citrate occurs prior to or during uptake. Although preliminary, these data are consistent with the system proposed by Sachan and Stern (20) in 1971 for the uptake of citrate by Enterobacter aerogenes and Salmonella typhimurium. In this system, a membrane-bound oxaloacetate decarboxylase acts additionally as a transport protein for citrate. Citrate, a competitive inhibitor of this enzyme, is thought to be transported from the cell surface to the inner membrane by this enzyme and is then cleaved by intracellular citratase to oxaloacetate and acetate. Subsequently, the oxaloacetate decarboxylase metabolizes oxaloacetate to pyruvate and CO_2. Experiments are planned to determine which enzymes are encoded on the cloned 9 kb Cit^+ DNA fragment.

Variable expression of plasmid-mediated citrate utilization. As mentioned above, our initial observation that the Cit$^+$ ability of strain V414 could be spontaneously lost at a high frequency (∿1%) led us to suspect that this Cit$^+$ ability is a plasmid-determined trait. Further study of this instability has, however, revealed a more complex picture of Cit$^+$ expression. Evidence has already been presented to show that in strain V414 the Cit$^+$ ability as well as genes for resistance to tetracycline and chloramphenicol are encoded by a 130 megadalton conjugative plasmid. Conjugal transfer of this plasmid, pWR60, can result in "en bloc" transfer of Cm^r, Tc^r, and Cit$^+$ ability. However, not mentioned above, when Cm^r or Tc^r was used as the selective trait to monitor pWR60 transfer, the resulting transconjugant strains were resistant to both antibiotics and contained a 130 Mdal plasmid, but appeared as citrate-nonutilizers (i.e., Cit$^-$). Following restreaking of these apparent Cit$^-$ transconjugants onto citrate minimal medium, Cit$^+$ colonies appeared at a low frequency (∿10^{-3} - 10^{-4} Cit$^+$ colonies/input cell). Similarly, the cloned citrate determinants in pWR61 have also been observed to alternate between a Cit$^+$ and a Cit$^-$ state of expression. Thus, it appears as though the plasmid genes involved in citrate utilization may be reversibly expressed, perhaps under the regulation of an invertible controlling element. Currently, studies are underway to demonstrate physically the existence of an insertion sequence element in the cloned Cit$^+$ gene region. Therefore, the apparent loss of Cit$^+$ ability in the original host strain, V414, now appears to be due in some cases to plasmid loss and in other cases to be a result of reversible expression of the Cit$^+$ trait.

II. Genetic Regulation of Reversible Vi Antigen Expression

Genetic characterization of the Vi antigen in Salmonella and Citrobacter. In *Salmonella typhi*, the Vi antigen is a surface component which may play a role in the virulence of this organism. This antigen is expressed as a stable genetic trait that is under the control of two widely separated chromosomal loci designated *viaA* and *viaB* (11,13,14,24) as shown in Figure 4. Certain strains of *Citrobacter* also produce the Vi antigen, but in these strains this surface antigen is expressed in an unstable state characterized by a reversible transition between cells exhibiting full Vi antigen expression and cells that appear not to produce the Vi antigen (25). Each cell type is thus reversibly able to generate the alternate cell type. This phenomenon is similar to that of flagellar antigen phase variation in *Salmonella*, the mechanism of which involves a DNA inversion that affects gene expression (22,29).

The studies described below represent a genetic analysis of the determinants controlling reversible Vi antigen expression in *Citrobacter freundii* (25). These preliminary genetic studies should provide suitable tools for planned molecular manipulations

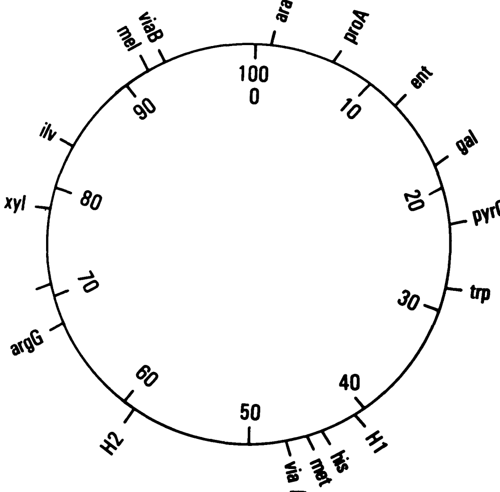


Figure 4. Chromosome map showing the location of certain Escherichia coli K12 genetic markers and pertinent Salmonella loci. The abbreviations used are defined elsewhere (1,21).

to determine if reversible Vi antigen expression is controlled by an invertible insertion sequence element similar to that involved in flagellar antigen phase variation (29).

Reversible Vi antigen expression in C. freundii. When cells of C. freundii strain WR7004 were streaked onto nutrient agar, two colony types were observed which are clearly distinguishable on the basis of their morphology. By examining individual colonies with a low power stereomicroscope under appropriate conditions of oblique lighting, the Vi antigen expressing forms appeared as bright, dense, orange colonies, that were clearly different from the dull, translucent colonies of the Vi-nonexpressing forms (Fig. 5). Furthermore, cells of colonies expressing the Vi antigen were agglutinated by specific Vi antiserum or lysed by Vi-specific phages. In addition to pure colonies of either type, variegated colonies containing both Vi-expressing and Vi-nonexpressing segments were frequently observed; a typical example of such a colony is shown in Figure 6. Upon restreaking a pure colony type, transition to the alternate colony type generally was found to occur at frequencies ranging from 5 to 50%. If colonies of either type which appeared to be pure (i.e., non-variegated) were serially restreaked, the state of the resulting colonies could generally be maintained in a nearly stable condition. It became evident from these studies, however, that each "pure" colony could contain considerably different numbers of transitioned cells of the opposite state, in spite of the stable appearnace of the colony. This finding

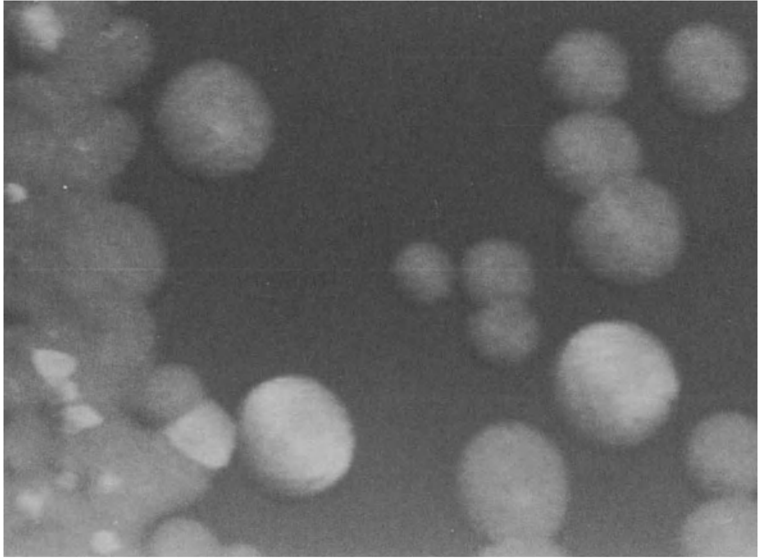


Figure 5. Colonies of *C. freundii* WR7004 showing reversible expression of the Vi antigen on nutrient agar. The Vi antigen is expressed by the light, shiny colonies, whereas expression of the Vi antigen is almost totally absent in the darker, dull colonies.

prevented us from determining more precisely the transition frequencies.

Transer to S. typhi of C. freundii locus controlling reversible expression of the Vi antigen. To transfer the locus involved in Vi antigen transition, an Hfr strain of *C. freundii* was constructed by mating *C. freundii* WR7004 with *E. coli* Hfr strain WR2014, and then selecting for receipt of the F-linked *lac*$^+$ genes of WR2014. The bacterial strains used in these and the following studies are listed in Table IV. The resulting *C. freundii* Hfr strain, WR7005, possessed the transfer orientation of WR2014, and was able to transfer its *viaB*$^+$ locus to a *mel*$^-$, *viaB*$^-$ derivative of *S. typhi*, strain WR4226, by selection for the closely linked *mel*$^+$ genes. Among Mel$^+$ transconjugants of WR4226 obtained from this conjugal transfer experiment, a number were observed to produce some colonies of a noticeably different morphology which resembled that of the Vi-expressing colony forms of *C. freundii*. These colonies were agglutinated by Vi antiserum and lysed by Vi phage. Upon restreaking, they gave rise to a mixture of Vi-expressing and non-expressing colonies. Continued restreaking of single colonies of either form resulted in the appearance of both forms. These results indicated that these Mel$^+$ *S. typhi* hybrids had acquired along with the *viaB*$^+$ locus of *C. freundii* all determinants necessary for reversible Vi antigen expression.

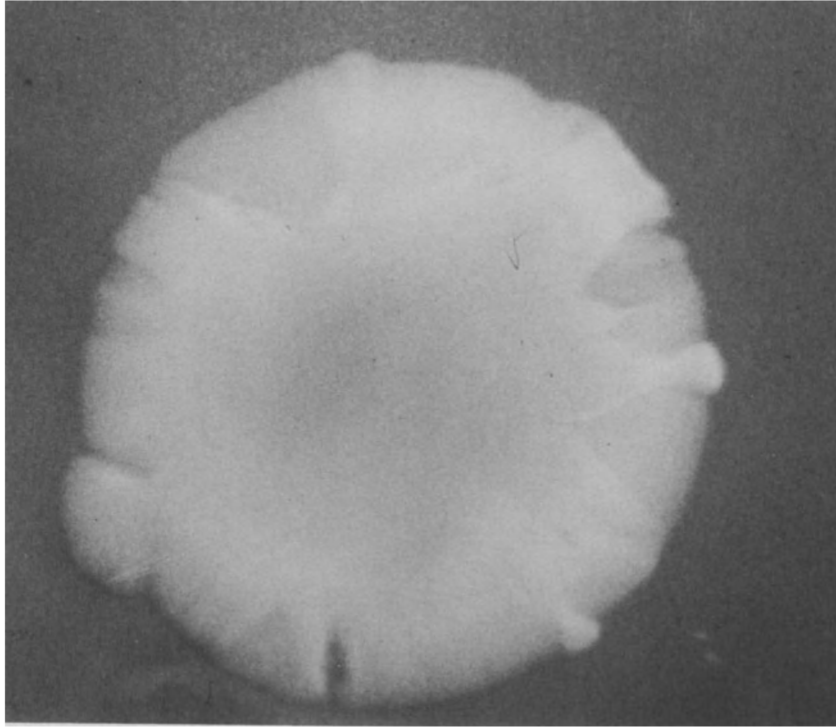


Figure 6. Variegated colony of C. freundii WR7004 exhibiting high frequency, reversible Vi antigen form variation on nutrient agar. The darker segments along the peripheral edge of the colony are Vi-nonexpressing forms. The majority of the colony is composed of Vi positive cells.

Presence of the $viaA^+$ locus in E. coli K12. In previous studies (14), it was shown that Salmonella typhimurium, although Vi negative, possesses a native, functional $viaA^+$ determinant and can express the Vi antigen after acquisition of the $viaB^+$ locus from S. typhi. Expression of the Vi antigen was also observed in a non-K12 strain of E. coli after transfer of the $viaB^+$ locus from S. typhi Hfr WR4000 (11). The presence of a native $viaA^+$ locus in E. coli K12 was established by transferring the closely linked his^+ region from E. coli Hfr WR2316, to S. typhi WR4205, a $viaA^-$,his^- recipient. Over 50% of the resultant his^+ S. typhi hybrids produced the Vi antigen. In addition, conjugal transfer of the viaB region of S. typhi Hfr strain WR4000 to the E. coli K12 recipient strain WR 2332 resulted in expression of the Vi antigen. These results confirmed the presence of a functional $viaA^+$ locus in E. coli K12. Although speculative, it seems likely that the viaA locus encodes some common property (e.g., cell surface structure) that is needed for Vi antigen expression.

TABLE IV
Bacterial Strains

Strain Designation	Description	Source
E. coli K12 WR2014	Hfr, metB, Str^s	P4X, KL227, B. Low from B. Bachmann
E. coli K12 WR2175	F^-, thr, leu, thi, mel, Str^r	From C600 hsdR, hsdM
E. coli K12 WR2316	Hfr, thi, Str^s	PK191, from B. Bachmann
E. coli K12 WR2332	F^-, proA, proB, mel	This study
C. freundii WR7004	Prototroph, Str^s	Formerly S. ballerup
C. freundii WR7005	Hfr, Str^s	WR2014 x WR7004 by terminal lac^+ selection
S. typhi WR4000	Hfr, Str^s	WR2014 x S. typhi 643 by terminal lac^+ selection (11)
S. typhi WR4205	ara, rha, fuc, xyl, cys, trp, met, his, lac, viaA, Str^r	Strain $643WS^r$ HM (25) spontaneous viaA mutant
S. typhi WR4226	ara, rha, fuc, xyl, cys, trp, mel, viaB, lac	viaB locus from S. typhimurium (25)
S. typhi WR4260	Hfr, cys, his, met, inl	This study; reversible $ViaB^+$ locus from WR7005

Transfer of the viaB locus of C. freundii to E. coli K12. Attempts to transfer directly the C. freundii $viaB^+$ locus to E. coli K12, using C. freundii Hfr7005, were unsuccessful. Although Mel^+ hybrids of E. coli K12 were obtained, none expressed the Vi antigen. To circumvent this problem, an S. typhi hybrid which had previously acquired the $viaB^+$ variable transition locus from C. freundii Hfr strain WR7005 was made into an Hfr strain. This S. typhi Hfr strain, WR4260, was derived by the F-linked terminal lac^+ selection method previously described. Subsequently, this donor S. typhi Hfr WR4260 strain was conjugally mated with E. coli F^- strain WR2175, and selection was made for Mel^+ hybrids. Over 90% of the resultant Mel^+ E. coli K12 exhibited a dense colonial morphology. Following purification on selective medium, the resulting dense colonies were streaked onto nutrient agar. Two clearly distinguishable colonial forms were observed using oblique lighting; a small, dense, shiny colony type with a distinctive orange color, showing some resemblance to the Vi expressing form of C. freundii; and a larger, dull, translucent colony resembling the K12 recipient. Further restreaking of single colonies of each type continued to yield mixtures of both colony types (Fig. 7). The shiny, orange-

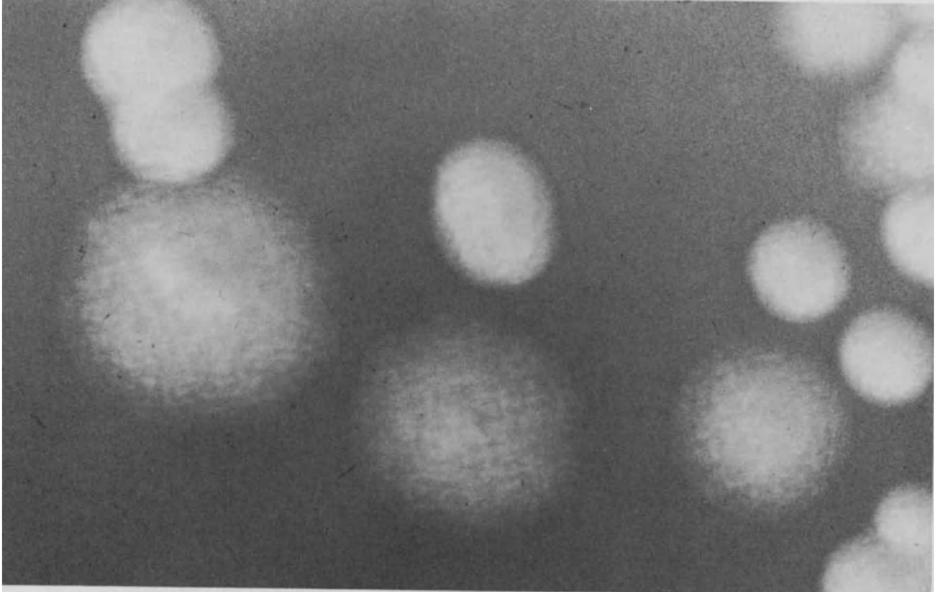


Figure 7. Colonies of E. coli K12 strain WR2376 containing the viaB$^+$ locus of C. freundii exhibiting variable Vi antigen expression on nutrient agar. The Vi antigen-expressing colonies are the small, lighter colonies. The larger, crinkled colony types are the Vi-nonexpressing forms. Restreaking each colony type results in a mixture of both forms.

colored colonies were identified as being Vi positive by their agglutination in Vi antiserum and by their sensitivity to Vi phage. Vi-positive and -negative colonies of these E. coli K12 hybrids were examined for acquisition of other markers from the S. typhi Hfr donor strain and for stability of the mel$^+$ marker. One Mel$^+$ K12 hybrid, exhibiting reversible expression of the Vi antigen, was selected for detailed study. As noted with most of the other hybrids, this strain, WR2376, had acquired the ara$^-$, tna$^-$, and xyl traits of S. typhi, but retained its native fuc$^+$ trait. The presence of these negative markers from S. typhi is an indication of haploidy and, hence, integration of at least these markers from this region of the S. typhi chromosome (12,15). On the other hand, when the mel$^+$ character of WR2376 was examined by streaking both Vi positive and negative colony forms on MacConkey melibiose medium Mel$^-$ segregants were readily observed. The mel$^+$/mel$^-$ diploid character of WR2376 was probably due to a tandem insertion (12,15) since plasmid DNA was not detected when the DNA of this organism was examined by the cleared lysate procedure. Segregation of the mel$^+$ locus did not affect the viaB$^+$ locus, i.e., Mel$^-$ colonies

exhibited reversible Vi antigen expression.

Influence of RecA gene product on reversible Vi antigen expression. Regulation of gene expression by an invertible controlling element has been observed to occur independently of the RecA gene product (i.e., general recombination) (22,29). Therefore, experiments were conducted to determine the effect of the *recA*$^-$ gene on Vi antigen expression in a strain exhibiting reversible Vi antigen transition. The *E. coli* K12 Hfr donor strain JC10240 was used to transfer the linked *recA*$^-$, *srl*$^-$, and Tc^r genes to recipient *E. coli* K12 strain WR2376. Tc^r WR2376 hybrids, some of which proved to be *Srl*$^-$, were tested for sensitivity to ultraviolet light and growth inhibition by nitrofurantoin (i.e., RecA$^-$ phenotype). All of the *recA*$^-$ WR2376 strains obtained were found to retain the ability to express the Vi antigen reversibly. These results indicate that the *recA* gene product is not required for reversible Vi antigen expression.

Transposition of the viaB locus from the chromosome to a plasmid. Physical characterization of the genetic "switch" mechanism governing reversible Vi antigen expression requires the isolation of these genes on a relatively small DNA molecule. Although recombinant DNA technology would appear to be the method of choice, no direct or indirect selection method could be devised to select for recombinant plasmids carrying the *viaB* locus. Therefore, we decided to use a temperature-inducible Mu phage to promote the transposition (6) of the linked Mel$^+$ region and *viaB* locus to an F'*lac* plasmid. *E. coli* K12 hybrid strain WR2376 was initially lysogenized with the temperature-inducible phage mu*cts*62 and, then was infected with an F'*lac*::mu*cts*62 plasmid. The resulting donor strain, WR2377, was conjugally mated at 37°C with an *E. coli* F$^-$ mu*cts*62 lysogen (strain WR2180; *mel*$^-$, *viaB*$^-$, *recA*$^-$), and Mel$^+$ hybrids were selected. All of the resulting Mel$^+$ hybrids retained the fermentation characters of the recipient WR2180 strain, which distinguished these hybrids from the donor strain. Six of the resulting 10^3 Mel$^+$ hybrids exhibited the orange color characteristic of Vi positive forms, when these colonies were examined by oblique lighting. When restreaked onto nutrient agar, these six hybrids generated colony types characteristic of reversible Vi antigen expression, as described previously. However, upon restreaking on MacConkey melibiose medium, Mel$^-$ segregants were occasionally obtained. These Mel$^-$ cells usually exhibited a total loss of Vi antigen expression, indicating that the *mel* and *viaB* loci are plasmid-determined. Of the six original *mel*$^+$, *viaB*$^+$ plasmid recombinants, three retained the Lac$^+$ phenotype of the parental F'*lac* plasmid, whereas the other three were Lac$^-$, probably due to insertion of the transposed genes into the *lac* locus of the F'*lac* plasmid. The DNA of these hybrid strains was subsequently examined. Each hybrid strain was found to contain a large plasmid of >100 megadaltons in size. Thus, the *viaB*$^+$ locus,

including the "switch" governing reversible Vi antigen expression, has been transposed to an F'lac plasmid. Current studies are aimed at employing these recombinant F'lac::mu plasmids as a source from which the viaB genes can be isolated, via recombinant DNA techniques, and transferred to a smaller plasmid.

SUMMARY

1. The atypical citrate-utilizing ability of two strains of E. coli has been shown to be plasmid-encoded. Strain V414 carries a 130 Mdal conjugative Cit^+ plasmid that also specifies Tc^r and Cm^r. Strain V517 carries 9 different plasmid species but only the 36 Mdal species is correlated with Cit^+ ability. These plasmids are different from previously reported Cit^+ plasmids of E. coli and Salmonella, which express thermosensitive conjugal transfer systems.

2. A 9 kb PstI fragment, carrying the Cit^+ genes of pWR60, has been cloned into the pBR325 plasmid.

3. Metabolic studies indicate that intact citrate is not incorporated directly into whole cells. Rather, atypical citrate utilization by these E. coli strains appears to involve partial metabolism of citrate at the cell surface before or during uptake.

4. The expression of atypical Cit^+ ability by the parental pWR60 plasmid or by the recombinant pWR61 plasmid appears reversible and may involve an expression switch mechanism (i.e., insertion sequence element).

5. Two widely separated genetic loci, viaA and viaB, are necessary for Vi antigen synthesis in Salmonella and Citrobacter. In some strains of C. freundii, Vi antigen expression is reversible, a phenomenon which can be visualized by a colonial morphology transition between Vi-expressing and -nonexpressing forms.

6. The C. freundii viaB locus appears to encode the Vi antigen as well as the genetic "switch" mechanism controlling reversible Vi antigen expression. The viaA locus, which is found in several different bacterial species, may encode some common property (e.g., cell surface structure or enzymatic activty) that is needed for Vi antigen expression.

7. S. typhi and E. coli K12 hybrid strains which carry the C. freundii viaB locus have been constructed. These hybrid strains express reversible Vi antigen expression, even in the absence of general recombination (i.e., functional recA gene product).

8. The C. freundii viaB locus was transposed via Mu-mediated events to an F'lac plasmid in the E. coli K12 hybrid strain WR2376. F' plasmids carrying the viaB locus should serve as a highly enriched source of viaB DNA for physical examination of the switch mechanism.

9. Genetic manipulations such as those described herein can be used to study virtually any plasmid, viral, or chromosomally-encoded property. The resultant better understanding of biochemical pathways and of genetic regulatory control systems, and the isolation of desired gene sequences should provide ample information and materials for improving chemical processes and constructing vaccines against various organisms.

ACKNOWLEDGEMENTS

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MOLECULAR CLONING IN THE STREPTOCOCCI

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INTRODUCTION

The genus Streptococcus contains a large number of species able to cause infection in humans and in animals. Our research efforts have revolved around the study of streptococci that normally reside in the human oral cavity. In this regard, Streptococcus mutans is thought to play an important role in the etiology of dental caries (tooth decay) in humans (5). Its virulence has been well established in animal model systems (see 5) and studies with mutants indicate that S. mutans' ability to synthesize water-insoluble glucans from sucrose is closely linked to its pathogenicity.

Data on the physiological and pathogenic properties of S. mutans continue to accrue at a rapid rate. Unfortunately, little is available in the way of genetic transfer systems in S. mutans. This severely limits our ability to analyze existing mutants. For this reason, about two years ago, we began to explore the feasibility of a streptococcal recombinant DNA system. We felt it crucial to develop a system that used a streptococcal host rather than a gram negative host in order to minimize problems with gene expression and gene product secretion. S. sanguis, one of the more common oral streptococci, was selected in this regard because it is able to develop genetic competence (transformability) naturally, without the use of physical agents or treatments.

In this report, we highlight the construction of a family of plasmid cloning vectors that can be used in our S. sanguis host system. In addition, we report on the development of a system termed the "helper-cloning" method (6) which must be used when cloning

chromosomal fragments from *S. mutans* in *S. sanguis*. This system allows for the recombinational rescue of incoming chimeric plasmids by a host cell resident plasmid that shares homology with the cloning vehicle.

MATERIALS AND METHODS

Bacterial Strains and Media

The strains of *Streptococcus sanguis* and *Streptococcus mutans* used in this work were cultivated routinely in Brain Heart Infusion ([BHI] Difco Labs, Detroit, MI) broth. Agar (Difco Labs, Detroit, MI) was added to BHI at a final concentration of 1.5% when solid medium was desired. Erythromycin or tetracycline was added to autoclaved media to give a final concentration of 10 μg/ml. All streptococcal strains were cultivated anaerobically.

Chemicals and Enzymes

Cesium chloride (technical grade) was obtained from Kawecki Berylco Industries, New York, NY. Agarose was purchased from Bethesda Research Laboratories, Rockville, MD. Lysozyme (grade I), protease (grade I), DNase I, RNase T1, and ethidium bromide, were obtained from Sigma Chemical Co., St. Louis, MO. Erythromycin was a gift from the Upjohn Co., Kalamazoo, MI. Tetracycline was obtained from Lederle Laboratories, Pearl River, NY. Restriction endonucleases and T_4 DNA ligase were purchased from Bethesda Research Laboratories. *Hind* III-digested phage lambda DNA was purchased from New England Biolabs, Beverly, MA.

DNA Isolation and Characterization

Plasmid DNA was isolated from streptococci by a previously published method (15). Alternatively, a modification of the Hansen and Olsen (9) method was used to obtain streptococcal plasmid DNA (J. Ash Tobian and F.L. Macrina, in preparation). Bulk cellular DNA was isolated as previously published (12). Estimation of plasmid copy number was performed as previously described (15). Restriction enzyme analysis and DNA ligase reactions were performed according to the manufacturer's instructions. Conditions for double digestion were as previously published (15). Electrophoretic analysis of DNA on agarose gels was performed according to the method of Meyers *et al.* (19). ^{3}H-thymidine labelling of streptococcal plasmid DNA and analysis of such material on neutral sucrose sedimentation velocity gradients was performed as previously described (12). Techniques for the visualization of plasmid molecules by electron microscopy were as previously described (12).

Transformation

The genetic transformation protocol of Lawson and Gooder (11) was used to introduce plasmid or chromosomal DNA into *S. sanguis*. Competent *S. sanguis* cells were exposed to DNA for 45 minutes at 37° C and the reaction was terminated by the addition of 50 μl of DNase I (10 mg/ml in sterile water) and 50 μl of sterile 0.1 M $MgSO_4$ to the reaction tube (culture volume was 0.33 ml). Transformation frequencies were expressed as number of transformants per viable recipient cell at the time of DNA addition.

Containment

The recombinant DNA experiments with streptococcal plasmids described herein fall into exemption category I-E-3 of the NIH Guidelines for Research Involving Recombinant DNA Molecules. The introduction of *S. mutans* chromosomal DNA into *S. sanguis* by "shotgun" recombinant techniques falls into exemption category I-E-4 of the NIH Guidelines (see Federal Register 45: 25368. 14 April 1980).

RESULTS

Plasmid Cloning Vector Construction

Our construction of streptococcal plasmid vectors has centered on the use of a phenotypically cryptic plasmid originally isolated in *Streptococcus ferus*. This plasmid, pVA380-1, is 2.8 x 10^6 daltons (2.8 Mdal) in size and is present to the extent of approximately ∿25 copies/chromosomal equivalent (13) in its natural host. pVA380-1 has been genetically transformed into *S. sanguis* by indirect selection and its copy number remains unchanged in *S. sanguis* (16). We reasoned that it would be possible to ligate gene sequences encoding directly selectable markers to the pVA380-1 replicon using recombinant DNA methodology. The marker we chose in this regard was erythromycin-resistance (Em^r) and our source of DNA bearing this trait was the previously characterized plasmid, pVA1 (14). pVA1 is a spontaneously-occurring deletion bearing derivative of the conjugative streptococcal plasmid pAMβ1. pVA1 is a 7.3 Mdal in size and confers Em^r at a level identical to that conferred by pAMβ1 (>200 μg/ml) pVA1 is non-conjugative, and is lost rapidly from host cells grown at 42°C but not from cells grown at 37°C (14). Restriction endonuclease cleavage site maps of pVA1 and pVA380-1 are seen in Figure 1.

We used a variety of strategies to link the Em^r gene sequence from pVA1 to pVA380-1. Both plasmids were cleaved simultaneously with *Eco* RI and *Hind* III, mixed in a ratio of 1:2 (pVA380-1:pVA1), ligated and used to transform *S. sanguis* V288 (plasmidless, standard

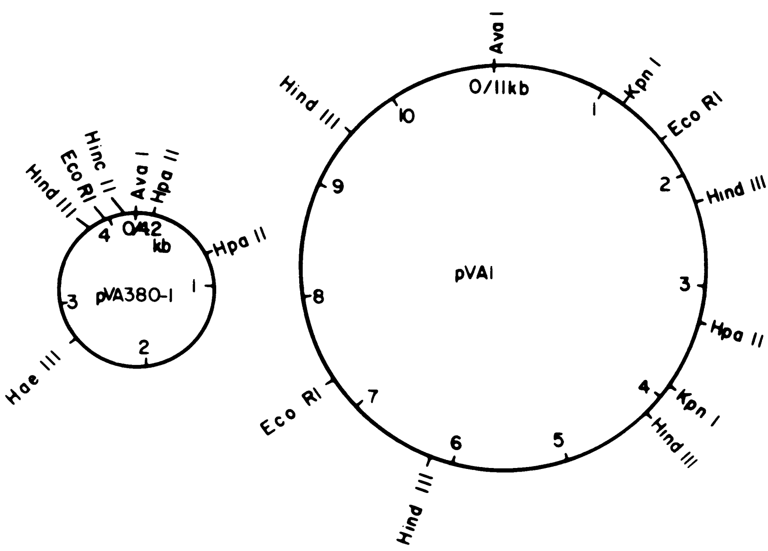

Figure 1. Restriction Endonuclease Cleavage Site Maps of pVA1 and pVA380-1. Relative position of sites was determined by analyses of fragments obtained following simulatneous-double digestions with all pairs of enzymes. Kb, kilo-base pairs. With only one exception, all of the enzymes listed on these maps yield cohesive termini upon cleavage. The Hinc II enzyme yields blunt ends.

host strain). Em^r transformants, recovered at a frequency of 10^{-4}-10^{-5}/recipient, were analyzed and found to contain the same recombinant plasmid, 5 Mdal in size. This plasmid, designated pVA736 (Figure 2), was subjected to restriction endonuclease site mapping and found to contain a Hind III-Eco RI fragment of pAV1 (approximately ∿9.5 kb to approximately ∿1.75 kb) linked to the largest Hind III-Eco RI fragment of pVA380-1. pVA736 was present to the extent of about 20 copies per chromosomal equivalent in S. sanguis.

Hind III-Ava I cleaved pVA380-1 and indentically cleaved pVA1 were mixed in a ratio of 1:2, ligated and used to transform S. sanguis to Em^r. Such transformants, obtained at a frequency of approximately ∿10^{-5}, were found to contain a 3.7 Mdal plasmid. One such plasmid was designated pVA738 (Figure 2) and was determined to consist of the Hind III-Ava I fragment of pVA380-1 (0-3.8 kb; Figure 1) linked to a Hind III-Ava I fragment of pVA1 (9.5-11 kb; Figure 1). pVA738 was present to the extent of ∿15 copies/chromosomal equivalent in S. sanguis.

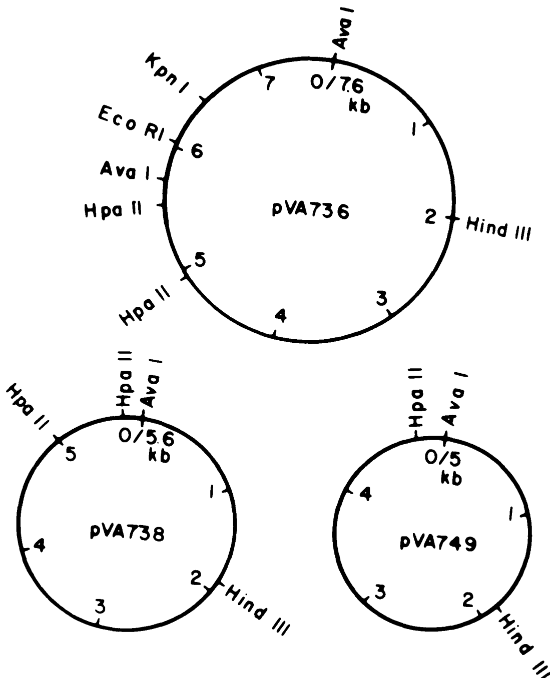

Figure 2. Restriction Endonuclease Site Maps of Streptococcal Vector Plasmids. See Table 1 for more information.

A final strategy employed in the construction of the pVA1: pVA380-1 family of vectors was to attempt to remove the Hpa II fragment (5-5.5 kb; Figure 2) from pVA738. This was implemented by completely digesting approximately ∿1 μg of pVA738 with Hpa II and allowing the linear Hpa II fragments to reanneal. Following ligation, this mixture was used to transform S. sanguis to Em^r. Transformants were obtained at a frequency of 10^{-5}-10^{-6} and 36 Em^r clones were examined physically for a plasmid that was smaller than pVA738. Only one such clone was obtained; the remaining clones contained a plasmid identical in size to pVA738. The one smaller plasmid was designated pVA749 and was 3.2 Mdal in size. The position of its single Hpa II, Ava I, and Hind III sites (Figure 2) indicated it to be the desired derivative of pVA738. pVA749 is a multi-copy plasmid but its exact copy number, as yet, has not been determined.

pVA736, pVA738, and pVA749 were stably maintained in S. sanguis even in the absence of selective pressure. This was true for cells grown at either 37°C or 42°C (<0.1% cells lost the Em^r marker under such conditions). None of these plasmids including pVA380-1, appear

to continued replicating in S. sanguis cells where protein synthesis has been blocked by the addition of chloramphenicol (15). The sites available on any of these plasmids for the insertion of passenger DNA are listed in Table 1. Information employed to determine the utility of such sites was derived from deletion mapping data using pVA1 (14) or from experiments where DNA was directly inserted into the site or sites in question (15; unpublished). Also included in Table I is information pertaining to pVA380-1. This cryptic plasmid may be used as a vector to clone any directly selectable gene (e.g., drug resistance markers from streptococcal R plasmids).

TABLE I
Restriction Endonuclease Sites Available for Inserting DNA into Streptococcal Vectors

Plasmid	Single sites into which passenger DNA may be spliced	Sites that may be used in combination
pVA736	Hind III, Eco RI, Kpn I, Ava I (replacement of 5.8-7.6 kb Ava I fragment) Hpa II (replacement of 4.9-5.6 kb Hpa II fragment)	Ava I-Hpa II Kpn I-Hpa II Eco RI-Hpa II Kpn I-Eco RI
pVA738	Hind III, Ava I, Hpa II (replacement of 5.0-5.5 kb Hpa II fragment)	Ava I-Hpa II
pVA749	Hind III, Ava I, Hpa II	Ava I-Hpa II
pVA380-1	Hind III, Eco RI, Ava I, Hpa II Hinc II[a]	Any combination of two of these enzymes.

[a]The Hinc II site on pVA380-1 may be used to insert passenger DNA using blunt end ligation.

Nature of Plasmid Transformation in S. sanguis

Preliminary experiments were performed to test the utility of pVA736 as a vehicle for introducing random S. mutans chromosomal DNA fragments into S. sanguis. pVA736 was cleaved using both Eco RI and Kpn I and mixed in a ratio of about 1:10 with similarly cleaved DNA from S. mutans 6715. Following ligation, this mixture was found to yield Em^r transformants at a frequency of approximately 1×10^{-6}. Sixteen Em^r clones were randomly chosen and examined for recombinant plasmids. In one representative experiment, six clones were found to contain plasmids the same size as pVA736, four contained plasmids

that were smaller than pVA736 and six contained plasmids that were larger than pVA736. To our surprise, not only had the smaller plasmids suffered deletions upon transformational entry in S. sanguis but one-half of the plasmids that appeared to be larger than pVA736 also had sustained deletions in the vector or inserted DNA as judged by restriction enzyme analysis (data not shown). Although deletion formation is not uncommon following transformation of plasmids into S. sanguis (14), we were struck by the high frequency of such events in our "shotgun" cloning experiments and decided to examine this system more closely.

^{3}H-labelled pVA736 DNA was prepared by CsCl-ethidium bromide centrifugation, and subjected to analysis on a neutral sucrose velocity sedimentation gradient. Following fractionation, the gradient was assayed for ^{3}H-radioactivity and biological transforming activity. The results are seen in Figure 3 and reveal nearly all of the ^{3}H-DNA (circles) to be monomeric pVA736 DNA (25S). A shoulder corresponding to high molecular weight forms (dimeric pVA736) is seen at fractions 20-23. The expected positions of dimer (33S), trimer (40S), and tetramer (45S) are noted on the figure. The profile of the transforming activity of the fractionated gradient (triangles) revealed most (>80% of total) of the transforming activity to be associated with oligomeric pVA736 forms. Samples of fractions 21-25 were pooled, ethanol precipitated and examined by electron microscopy. Such analysis revealed that dimers (head-to-tail) made up approximately 8% of the total molecules (n = 116) while no dimers were seen in material examined from fractions 14-19 (n = 135; only monomers were seen in this fraction pool). No plasmid DNA molecules could be visualized from fractions 26-30 by electron microscopy, despite exhaustive screening. This suggests that the material in the less than 35S range has high biological activity. Components of pVA736, corresponding to dimers (or other oligomeric plasmid forms), were never detected as discretely staining bands on agarose gels even when such gels were deliberately overloaded with CsCl-ethidium bromide purified pVA736 DNA. With regard to biological activity, cells that were transformed with pVA736 DNA from any portion of the gradient seen in Figure 3, always were found to contain monomeric pVA736 upon physical analysis.

The transforming properties of purified monomeric pVA736 DNA were examined by kinetic analysis. Electrophoretically purified monomeric pVA736 DNA was used to determine a dose response curve. As controls, the transforming activities of CsCl-ethidium bromide purified pVA736 DNA (containing naturally-occurring oligomeric forms) and chromosomal DNA from a rifampicin resistant S. sanguis strain were similarly determined. Figure 4 is a log-log plot of the data obtained in these experiments. The slopes of the curves obtained with the chromosomal and CsCl-ethidium bromide purified DNAs were about 1, while that obtained using the purified monomeric pVA736 DNA was about 2. We interpret the monomer derived curve to mean

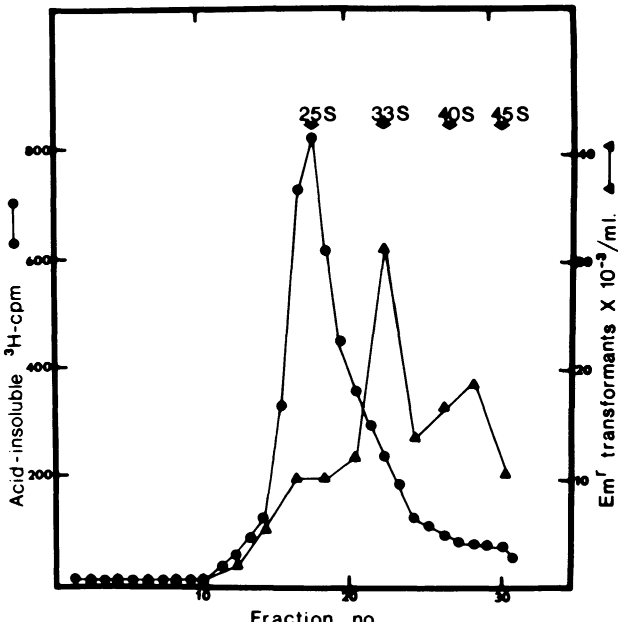

Figure 3. Velocity Sedimentation Analysis of pVA736. CsCl-ethidium bromide purified pVA736 DNA (~0.3 μg ^{3}H-DNA + ~3 μg of unlabelled DNA) was layered onto a 5-20% neutral sucrose gradient. Sedimentation was for 160 min. at 42,500 rpm in a Beckman SW50.1 rotor at 15°C. Four drop fractions were collected by piercing the tube bottom with an 18 ½ gauge needle. Fifty μl of each fraction was assayed for ^{3}H-DNA; 50 μl of alternate fractions was assayed for its ability to transform competent S. sanguis to Em^r. Finally, the fractions indicated in the text (14-19; 21-25; 26-30) were pooled, ethanol precipitated and prepared for electron microscopy. Sedimentation was from left to right.

that two monomeric pVA736 molecules were needed to yield a viable transformant. On the other hand, the slope of the CsCl-ethidium bromide purified DNA suggested that the transforming activity of this material, at the concentrations used, derived from oligomeric pVA736 molecules that were inferred to transform with one-hit kinetics. This notion is consistent with the significant amount of transforming activity associated with pVA736 oligomers (Figure 3). Taken together these data are similar to those obtained by Saunders and Guild (7,22,23; in press, J. Bacteriol.) using S. pneumoniae. These authors have proposed a model to explain these data that is based on the low efficiency assembly of single stranded plasmid fragments which have separately entered a competent streptococcal cell. This will be discussed below.

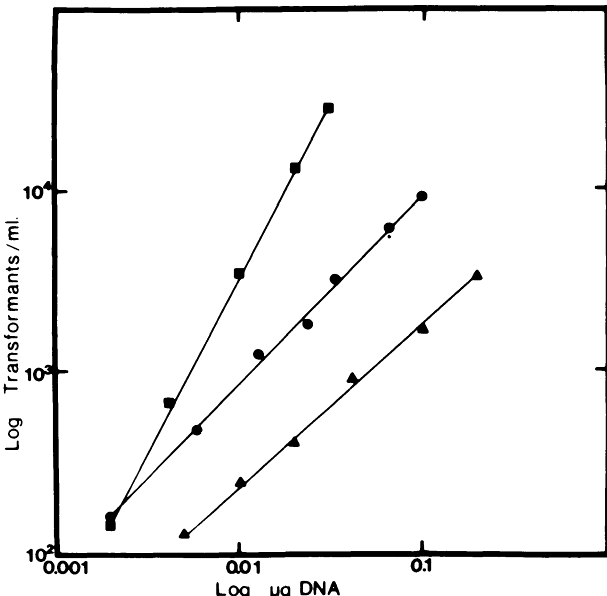

Figure 4. Transformation of S. sanguis (Challis) with Plasmid and Chromosomal DNA. Circles: dye-buoyant density purified pVA736. Squares: purified monomer pVA736. Triangles: chromosomal DNA prepared from rifampicin-resistant S. sanguis. DNA was exposed to competent cells for 30 min, followed by 5 min. of treatment with pancreatic DNase I (10 μg/ml). Cells then were plated directly on selective medium (Brain Heart Infusion Agar [Difco, Detroit, MI]) containing 10 μg erythromycin/ml or 25 μg rifampicin/ml.

Development of a "Helper Plasmid" Method for Molecular Cloning

Our data relative to the kinetics of plasmid transformation raised serious questions about the efficiency of "shotgun" cloning of chromosomal DNA into S. sanguis. The formation of multimers carrying a given chromosomal fragment seemed remote due to the large size of the chromosome. Similarly, the formation of large numbers of monomers of a given chimera, needed to give two-hit kinetics, seemed very unlikely. To circumvent this problem, we adapted to our system the "helper plasmid" cloning method of Gryczan et al. (6). This method, originally developed for use in B. subtilis, obviates the requirement for multimeric plasmid forms in transformation. It employs a host that contains a resident plasmid that shares homology with the vector being used. This system allows for the recombinational rescue of incoming chimeras even if such chimeras are not oligomeric in nature.

Our control experiments to test this system used pVA736 as a donor plasmid (Figure 2) and three isogenic *S. sanguis* hosts. *S. sanguis* V288 contained no plasmid DNA, V685 contained pVA380-1, and V679 contained pVA380 (2.6 Mdal). The 2-6 kb region of pVA736 is homologous to pVA380-1 (Figures 1 and 2 and related discussion) while pVA736 shares no homology with pVA380. Uncleaved pVA736 or pVA736 cleaved with *Hpa* II were used to transform each of the three hosts. These results, (Table II), indicated that the pVA736, linearized with *Hpa* II, poorly transformed the plasmidless host (V288)

TABLE II
Transformation of *S. sanguis* With *Hpa* II Cleaved pVA736 DNA

S. sanguis host	Plasmid	Hpa II[a] Uncleaved	Hpa II[a] Cleaved
V288	NONE	2.9×10^{-2}	1.2×10^{-6}
V685	pVA380-1	2.7×10^{-2}	3.6×10^{-3}
V679	pVA380	8.0×10^{-3}	9.0×10^{-7}

[a]Cells were transformed with 0.5 μg of cleaved or uncleaved DNA as noted. Transformation conditions were as described in the legend to Figure 4.

and the non-homologous plasmid containing host (V679). The few transformants recovered in these experiments contained intact pVA736 DNA and we believe these colonies originated from cells that were transformed with plasmid molecules that escaped *Hpa* II cleavage. However, *Hpa* II cleaved pVA736 DNA was able to transform V685 at an efficiency that was within one order of magnitude of the frequency obtained with uncleaved plasmid DNA. A limited number of transformants from such experiments were examined for plasmid DNA and revealed the following information. First, Em^r transformants of V685 never contained pVA380-1. This was expected because the newly formed Em^r recombinant molecule would be incompatible with pVA380-1. This latter molecule would be rapidly lost in the face of the selective pressure of the Em. Second, the recombinant plasmids obtained in such rescue experiments were identical to pVA736 when analyzed by restriction enzyme cleavage. This indicated that the recombinational rescue of the *Hpa* II fragment of pVA736 by pVA380-1 restored an intact pVA736 molecule. This was also predicted from the expected pairing of the pVA736 *Hpa* II fragment with the resident pVA380-1 molecule. This hypothetical paired intermediate is seen in Figure 5.

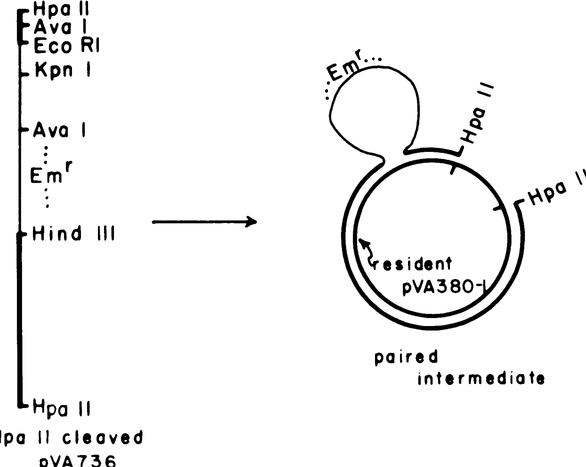

Figure 5. Hypothetical Paired Intermediate Between Hpa II Cleaved pVA736 and a Resident pVA380-1 molecule. pVA380-1 sequences are depicted as thick lines; non-pVA380-1 sequences are depicted as thin lines. The relative location of the Em^r determinant on pVA736 has been noted.

This rescue strategy was further tested as follows. pVA736 DNA that had been cleaved simultaneously with Ava I and Hpa II (Figure 2) was mixed with a ten-fold excess of similarly cleaved S. mutans 6715 DNA. Following ligation, half of the mixture was used to transform S. sanguis V288 (plasmidless) and the other half was used to transform S. sanguis V685 (containing pVA380-1, which shares homology with pVA736). Em^r transformants were detected using V288 at a frequency of 1×10^{-6} while such transformants occurred at a frequency of 1×10^{-4} in the V685 host. Furthermore, about 75% of all Em^r transformants obtained in the V685 background contained S. mutans inserts. The inserts tended to be small in size (0.5 to 2.0 Mdal) but this was due to the fact that Ava I-Hpa II digestion of the S. mutans chromosomal DNA renders fragments predominantly in the small size range. Em^r transformants obtained using the V288 host contained pVA736. Again we assume these to represent transformants that arose following entry of pVA736 DNA that escaped the original endonuclease cleavage.

Recently, we have successfully applied this "helper plasmid" cloning methodology to isolate a S. mutans chromosomal sequence that confers high level tetracycline resistance (J. Ash Tobian and F.L. Macrina, in preparation). This marker was easily cloned due to its direct selectability. However, the success of this experiment

indicated that "shotgun" cloning of specific heterologous chromosomal DNA in the *S. sanguis* host is possible using the "helper plasmid" method.

DISCUSSION

The construction of plasmid cloning vehicles for use in a streptococcal recombinant DNA system has been relatively straightforward. As can be seen in Table I, passenger DNA can be added to our vectors using several restriction enzymes alone or in combination with one another. All of our vectors are stable in *S. sanguis* and are present as multicopy plasmids. Unfortunately, none of these plasmids appear to replicate in the absence of protein synthesis and, thus, are not amplifiable. Because pVA736, pVA738, and pVA749 encode only one drug resistance marker, they do not afford the opportunity to use insertional inactivation strategies (24). Streptococcal chimeric plasmids that do contain two resistance markers have been reported, however (7,15,17). Such plasmids need to be examined for their potential use in cloning *via* insertional inactivation (i.e., unique restriction site occurring in one of the two drug resistance markers). Table III is a compilation of some potentially useful cloning vehicles, that have been constructed or characterized in laboratories other than ours. Clearly, the area of streptococcal cloning systems is experiencing rapid growth presently, and this will hopefully result in providing a number of useful vector and host systems.

TABLE III
Other Potential Streptococcal Plasmid Cloning Vectors

Plasmid	Size (Mdal)	Copy No.	Marker(s)	Single Cleavage Sites	References
pVA318	3.6	30	None	*Hind* III[a] *Pst* I[b] *Hae* III[b]	2,8
pDB101	11.9	6	Em^r	*Hpa* II[a] *Eco* RI[a] *Kpn* I[b]	1
pMV158	3.4	50	Tc^{rc}	*Hind* III[b]	3,7 V. Burdett, pers. comm.
pSM10	5.4	multicopy	Em^r	*Ava* II[a] *Eco* RI[b] *Ava* I[b] *Kpn* I[b] *Hpa* I[b] *Pvu* II[b]	17,18

TABLE III
(Continued)

Plasmid	Size (Mdal)	Copy No.	Marker(s)	Single Cleavage Sites	References
pSM10221	8.5	multicopy	$Em^rCm^{r\,d}$	Ava II[a] Ava I[b] Kpn I[b] Hpa I[b] Pvu II[b] BstE II[b]	17

[a]Site can be used to ligate DNA to plasmid as indicated by direct demonstration or deletion mapping.
[b]Data not yet available on utility of site for cloning purposes.
[c]Tc^r = tetracycline resistance.
[d]Cm^r = chloramphenicol resistance.

The anomalies associated with the cloning of *S. mutans* chromosomal fragments in *S. sanguis* (i.e., excessive deletion formation) forced us to study the nature of the transformation process as it pertained to plasmid DNA. The work of Saunders and Guild (7,22,23) was helpful in this regard. These authors had previously shown that purified monomeric plasmid DNA transformed competent *S. pneumoniae* by a process that followed second order kinetics while multimeric plasmids transformed with first order kinetics. Saunders and Guild (7,22,23; *J. Bacteriol.*, in press) have advanced a model to explain these data. In *S. pneumoniae* (as in *S. sanguis*) the entry of chromosomal DNA fragments into a competent cell involves the degradation of one strand of the molecule with only a single strand actually entering the cell (20,21). Nucleolytic shortening of such incoming single stranded fragments is also known to occur. This process likely occurs during plasmid transformation in streptococci. In fact, Lacks (10) has shown that circular bacteriophage DNA is nicked upon binding to competent pneumococcal cells and is converted to single stranded DNA upon entry into the cell. The Saunders and Guild model rationalizes that two monomeric single stranded plasmid molecules must enter the cell and that these donor molecules have base sequence overlap in order to facilitate recircularization. This accounts for the observe two-hit kinetics. Single stranded oligomeric molecules enter the cell and are envisioned to recircularize *via* a recombination-repair event. A second small plasmid fragment may be required for the regeneration of monomers by this process. If so, it must enter at the same time as the major donor fragment, in order to be consistent with the one-hit kinetics observed for oligomeric forms.

The kinetic analyses obtained using the S. sanguis plasmid transformation system (Figure 4) are consistent with the Saunders and Guild model. The data, in any event, raise serious questions about the efficiency of molecular cloning in such a system. Cloning experiments involving two plasmids, one as vector the other as passenger DNA, appear feasible if ligations are done at relatively high DNA concentration. Under such circumstances, the formation of multimers of the desired recombinant would be favored. Alternatively these conditions would also favor the formation of significant numbers of the same monomeric chimera needed to effect two-hit kinetics. The fact that we have been so successful in constructing plasmid-plasmid chimeras supports this view.

We feel that the initial problems (i.e., extensive deletion formation) seen in attempting chromosomal shotgun cloning in the S. sanguis system may be traced to these considerations. Due to the large size of the host chromosome the formation of multimeric chimeras, or large numbers of an identical monomeric chimera, would be remote.

The helper-cloning method initially devised in Dubnau's laboratory provides one solution to this problem (6). Dubnau's group has used this method to clone chromosomal erythromycin resistance determinants in Bacillus (4). In essence, this method greatly increases the efficiency of transformation by allowing incoming chimeric plasmids to be rescued by a resident homologous plasmid. This obviates problems with replicon reassembly from single stranded fragments. Our data clearly show this strategy to be a viable one in S. sanguis. Our cloning vehicles all were derived from the pVA380-1 replicon. This cryptic plasmid has been introduced into S. sanguis by indirect selection (16). This pVA380-1-containing strain makes an ideal host in the "helper plasmid" cloning system. The plasmid is multicopy in nature and bears no drug resistance markers. At this point we are not sure of the size range of fragments that may be rescued by recombination with pVA380-1. A tetracycline resistance determinant cloned from a S. mutans strain using the helper method was found on a fragment that was approximately 4 Mdal in size (J. Ash Tobian and F.L. Macrina, in preparation). Further work is needed in order to determine the maximum fragment size that may be cloned by this method.

An alternative solution to performing shotgun chromosomal cloning in S. sanguis is to first build a library of desired chromosomal fragments using a conventional Escherichia coli recombinant DNA system. This would provide one with a chromosomal fragment in relatively pure form as part of an E. coli derived chimera. This fragment could than be readily subcloned into S. sanguis using any of the streptococcal vectors described here. We have recently constructed a plasmid vector that is able to replicate in and express drug resistance markers in both E. coli and S. sanguis. We expect

that this "shuttle" vector will greatly facilitate this latter approach to doing recombinant DNA research in the streptococci.

ACKNOWLEDGEMENTS

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DISCUSSION

Q. What are the molecular events in your marker rescue system; the role of single strands, the role of double recombination between circles?

A. MACRINA: We cannot be sure of the exact molecular events that are operative in the recombinational rescue. The dimer molecule is probably single stranded based on what we know about the transformational uptake of DNA in S. sanguis. Whatever the recombinational steps involve, they seem to work with great efficiency.

GENETIC ENGINEERING ON MICROORGANISMS FOR CHEMICALS:

DIVERSITY OF GENETIC AND BIOCHEMICAL TRAITS OF PSEUDOMONADS

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INTRODUCTION

The genus Pseudomonas includes both saprophytes and species pathogenic to plants and animals. In the context of this volume, the most important attribute must be in the very wide variety of chemical transformations which individual strains can perform, as a result of their important ecological role in mineralization of organic matter.

Pseudomonas strains are extremely common, and are often the predominant members of the populations selected from natural sources such as soil, polluted waters, sediments etc. for their ability to grow on single compounds as sole carbon or carbon and nitrogen sources. This is particularly true when the selective enrichment is carried out as an aerated batch culture with relatively high levels of substrates: this is probably less true of chemostat enrichments in which other saprophytic bacterial genera often predominate. Pseudomonas spp. have been reported as being capable of growing upon a very wide range of chemicals including alkanes, mono- and polycyclic hydrocarbons, alicyclics, heterocycles, phenolics, aliphatic and aromatic halogenated compounds, terpenes, flavonoids, etc., the list is almost endless. In addition to the utilization of these compounds as growth substrates, many enzymes of these pathways have a wide specificity which can allow their use for the partial metabolism of compounds related in structure to the growth substrate, but which cannot support growth. This has been called co-oxidation (50) and is one way of modifying chemicals which themselves are poorly degraded or not degraded at all. Such non-specificity of metabolism has also been put to use to select

for mutants blocked in catabolic pathways where the defective mutant survives the exposure to a substrate analogue.

Pseudomonas strains are essentially aerobic, although some can use nitrate as a terminal acceptor for a limited number of substrates Some strains are classified with phototrophs and these are facultatives (Kaplan, this volume). The aerobic catabolic pathways for the more reduced and hydrophobic substrates are oxidative and usually involve the direct incorporation of molecular oxygen into a metabolite by mono- or dioxygenase reactions. However, a wide variety of other enzymatic reactions are also found particularly when the substrates carry substituents not usually associated with general metabolism, such as halogen atoms, nitro- or sulphonate groups which cannot be eliminated before the carbon skeleton can be converted to central metabolites.

Individual strains of *Pseudomonas* very often have a wide range of potential growth substrates: the definitive taxonomic study of Stanier *et al*. (63) lists strains capable of utilizing nearly 100 different chemicals. This suggests that considerably more of the *Pseudomonas* genome may be involved with catabolism than, for example, that of the *Enterobacteraceae*.

However, growth on substrates, which are often described as esoteric, is found in only a very few strains and these usually are isolated by selective enrichments. This presents a difficult task for the geneticist interested in the peripheral catabolism of this genus, because of its great taxonomic, and therefore genetic heterogeneity.

TAXONOMY OF PSEUDOMONAS

The relationships within the broad spectrum of strains classified as *Pseudomonas* was investigated by Stanier *et al*. (63) and has been reviewed by Palleroni (49). The genus falls into five main groups by the criterium of ribosomal RNA homology (Table I); the survey as Palleroni (49) indicated does not include a number of strains of marine origin described by Bauman *et al*. (7) many of which have been assigned to another genus (*Alteromonas*), largely on the basis of G+C content of their DNA.

Group 1 contains the three fluorescent pigment-producing species *P.aeruginosa*, *P.putida* and *P.fluorescens* as well as non-fluorescent species *P.stutzeri* and *P.alcaligenes*. Of the entire genus the only significant genetic studies have been conducted with *P. aeruginosa* and to a lesser extent *P.putida*. Other species which have been found to have interesting catabolic abilities are the non-fluorescent *P.cepacia* (originally *P. multivorans* because of its

TABLE I

Classification of Pseudomonas Species into RNA Homology Groups

rRNA homology group	GC in DNA (%)	Species[a]
1	67	P. aeruginosa (131)
	59 - 63	P. fluorescens (D-31)
	61 - 62	P. putida
	58 - 61	Fluorescent plant pathogens
	61 - 66	P. stutzeri
	63 - 64	P. mendocina
	66	P. alcaligenes
	63	P. pseudoalcaligenes
2	67 - 68	P. cepacia (382)
	68	P. marginata
	65	P caryophylli
	69	P. pseudomallei
	69	P. mallei
	64	P. pickettii
	66 - 67	P. solanacearum
3	67	P. acidovorans(14)
	62	P. testosteroni
	65 - 66	P. delafieldii (134)
	62 - 64	P. facilis
	69	P. saccharophila
4	66 - 67	P. diminuta (501)
	66	P. vesicularis
5	67	P. maltophilia (67)
	66 - 68	Xanthomonas spp. (Xc-1)

[a] Taken from Palleroni (1975)

catholic appetite) in Group II, and P.acidovorans and P.testosteroni in Group III. Some interesting work has been done on the catabolic pathways and their regulation in these species highlighting the variety of metabolic and regulatory potential available. Thus, for example, P.fluorescens and P.cepacia degrade tryptophan *via* the aromatic pathway with catechol as an intermediate whereas P.acidovorans uses a different route, the quinoline pathway with kynurenate as an intermediate (63). Group I organisms usually utilize aromatic compounds such as benzoate via catechol and the *ortho* (or β-ketoadipate) pathway, whereas Group III strains use a biochemically different pathway (Figure 1). Although *p*-hydroxybenzoate is metabolized to protocatechuate by Groups I and III, *ortho*- and *meta*-pathways are used for the generation of amphibolic intermediates, respectively (Figure 2).

COOH
A
B
HO COOH
OH
COOH
OH
OH
COOH
OH
OH
OH
1,2-ortho
or
2,3-meta
ring cleavage
4,5-meta

Figure 1. Catabolism of benzoate by (A) Group I and (B) Group III, pseudomonads.

These particular features appear to be highly conserved within the species. This has been further demonstrated by the regulatory constraints used for the pathways (62) and the homologies of isofunctional enzymes revealed by immunological methods (16, 64). However, many of the catabolic pathways of the compounds peripheral to central metabolism appear to be distributed thinly and at random amongst the species and, to add to the complexity, it is the experience of many laboratories, who have selectively enriched an interesting strain from nature, that it does not fall into any of the described species and must be described either as being an atypical species or even just as *Pseudomonas sp*. It would appear that the genus represents a large number of distinct species with perhaps even a continuum of strains the genetics of which represent an almost total wilderness of scientific ignorance. The range of strains in nature might be likened to a complement (*sic*) of plasma cells producing antibodies to diverse antigens. Having isolated an indeterminate *Pseudomonas sp*. with interesting catabolic features, the geneticist is faced with the options of either beginning a study of the genetic organization of a pathway in an uncharacterized host background, or transferring (cloning) the genes of

Figure 2. p-Hydroxybenzoate catabolism by (A) Group I and (B) Group III pseudomonads.

interest into a genetically more amenable host, or both. The transfer to genetically characterized hosts could be accomplished by *in vivo* or *in vitro* techniques. This requires that the catabolic genes are expressed for selection, or that a co-selectable neighboring marker be introduced prior to the transfer.

GENETICS OF PSEUDOMONAS AERUGINOSA

What is known about the genetics of *P.aeruginosa* have been thoroughly presented in a number of reviews (29, 31, 33, 34). Two *P.aeruginosa* strains have been studied in some detail, PAO and PAT The two main techniques used for study of the chromosomal genetics of these strains have been transduction and conjugation. Among a number of phages which propagate on *P.aeruginosa*, F116L and G101 (33) have been used as generalized transducing phages and they

transfer markers at frequencies of between 10^{-7} - 10^{-6}. No specialized transduction has been reported, although it seems likely that all P.aeruginosa strains are lysogenic for at least one phage.

Conjugal transfer of chromosomal genes requires the existence of plasmids with what Holloway has described as Cma (chromosome mobilizing ability) (Table II). Several such plasmids have been used in PAO and PAT. Plasmids FP2, FP5 and FP110 can be used in interrupted matings and plate matings. Each transfers chromosome from a single origin, FP2 and FP5 from the same origin and FP110 from a point about 27 min. from the FP2/5 origin and in the opposite direction. In strain PAT three sex factors have been used FP2-2 (derived from FP2), R68 and R91-5. The first two transfer from single but different origins, and the latter from two origins. Maps have been published for the two strains, for about 90 loci for strain PAO and about 50 for strain PAT (31, 34) and both have now been shown by genetic linkage to have circular chromosomes. One of the most interesting and useful offshoots of these studies was the procurement of the derivative plasmid R68-45 from R68 by selection for an increased frequency of chromosome mobilization. This was isolated as a result of transfer of R68 from PAO into an auxotrophic PAO recipient, a cross normally of very low frequency. If the selection is for certain areas of the chromosome (five have been identified, 32), plasmids such as R68.45 can be found in the transconjugants. R68.45 mobilizes the chromosome from multiple origins in PAO, PAT, P.putida strains and has been a vital tool in demonstrating the circularity of the PAO chromosome (59, 60).

Because it is of the promiscuous Pl incompatibility group, it will transfer to a wide range of gram negative genera, and has been successfully used as a sex factor in them as well (31). Physical studies have shown that R68.45 is larger than R68 by 2.1 Mdal. It was earlier thought that its acquired DNA was from the chromosome of PAO, and that interaction of this DNA with various sites on the chromosome lead to its enhanced chromosomal mobilization (Holloway, 1979). However, recent physical mapping has shown that the additional DNA is the result of tandem duplication of 2.1 kb of R68 DNA (N. Willetts, pers. comm. and 56). The mechanism by which this duplication leads to its enhanced chromosome mobilization, not only in P.aeruginosa but also in the other genera has yet to be elucidated.

Formation of R' Plasmids

Whereas transduction and conjugation are of use in mobilizing genes between strains when the genes can recombine in the recipient cell, a more general method of transferring genes between nonhomologous strains is possible if those genes can be covalently linked as part of a transmissible plasmid, analogous to the F' plasmids

TABLE II

Plasmids with Chromosomal Mobilization Ability in *Pseudomonas*

Plasmid	Host range Cma	Origins	Direction	Other comments
FP2	PAO PAT	One	Clockwise	Carries Hg^R determinant
FP39	PAO	One	Clockwise	
FP5	PAO	One (same as FP2)	Clockwise	Cured with acriflavin
FP110	PAO	One	Anti-clockwise	Gives FP' plasmids
R68	PAT	One	Clockwise	P-1 Incompatibility group Efficient Cma (10^3/donor)
R91-5	PAT PAO Acinetobacter	Two	?	P-10 Incompatibility group.
R68.44	PAO	Multiple	?	Enhanced Cma in PAO R68.45 more stable than R68.44 Wide host range
pND2	PAO	Multiple	?	Isolated from *P.arvilla* (*putida*) mt-2; carries TOL functions.
XYL-K	*P.putida*	One	?	K originated from the OCT plasmid.
pfdm	*P.putida*	?	?	Derived from phage pf16h2

* Examples taken from Holloway (1979).

of the enteric bacteria. This has been demonstrated possible by Hedges et al. (28) and by Holloway (30). Both used derivatives of R68 in P. aeruginosa donors which they mated into auxotrophic recipients in which the transferred chromosomal genes could not recombine. However, whereas Holloway (30) used a recA isogenic P.aeruginosa as recipient, Hedges et al. (28) used an E.coli recipient; a similar R' plasmid pMO74 has been constructed using R68.45 transfer into an arg⁻ P.putida strain (P.H. Clarke, pers. comm.). The amount of chromosomal DNA incorporated in these three plasmids appears to be large, from 60 - 80 Md (P.H. Clarke, pers. comm.) and obviously incorporates a large number of other genes in addition to the marker selected in the original mating. So far direct selection for the formation of R' plasmids carrying catabolic genes does not appear to have been successful although pMO74, which was selected to carry arg genes also carries the genes for amide catabolism which are closely linked. Obviously this method of in vivo cloning has distinct potential for the transfer of catabolic genes between species given the promiscuity of R68.45 and some other Inc P plasmids. One limitation to its potential would be if the constituent genes for chromosomally encoded catabolic pathways are separated by distances larger than can be integrated into a single R' plasmid. As yet there is very little experimental evidence as to whether this is the case. Certainly there is some evidence from cotransduction with phage pf16h2 (15) that there is clustering of the mandelate genes (57, 58), and the β-ketoadipate pathway genes (39, 69) and other catabolic genes (66) in P.putida. Using the same phage Leidigh and Wheelis (41, 42) have further indicated that there may well be a supraoperonic clustering of catabolic genes concentrated at a region of 10-15% of the chromosome. However confirmation of this probably requires further genetic and physical evidence before anything more than tentative conclusions can be drawn. Even on catabolic plasmids such as OCT and TOL, where one might expect there to be selection for clustering for co-ordinated genes, there appears to be surprisingly large distances between the constituent genes of a particular pathway (Shapiro, this volume, and 26, 36).

CATABOLIC PLASMIDS

The initial discovery that naturally occurring plasmids existed which carried genes responsible for catabolic pathways was first made in strains of Pseudomonas and at the host institution of this conference, the University of Illinois, in the laboratory of I.C. Gunsalus. The first of these described coded for the catabolism of camphor (CAM) (54). Discovery of plasmids for salicylate (SAL) (12) naphthalene (NAH) (23), and octane (OCT) (13) degradation, was rapidly followed by the independent discovery in three laboratories of a plasmid TOL for the degradation of toluates (47, 71, 73) and later shown to encode the complete pathway for breakdown

of toluene , and several alkyl substituted derivatives *m*- and *p*-xylene (40, 72a, 75, Kunz and Ribbons, unpublished). Since then there have been a number of other reports of catabolic plasmids responsible for the breakdown of substrates as diverse as nicotine (65), 3,5-xylenol (35), 4 methyl-o-phthalate (1,2) the herbicide 2,4-dichlorophenoxyacetate (25) and the detergents alkylbenzene sulphonates (9). The possession of catabolic plasmids does not appear to be the sole prerogative of *Pseudomonas* and they have been demonstrated in other genera including *Alcaligenes* (25) *Agrobacterium* (20, 44) and Micrococcus (23a). It has been the contention of one of us (PAW) for several years (72), that many of the more exotic catabolic pathways in the saprophytic microbial population will eventually be demonstrated to be plasmid coded, and their existence may well be one of the major reasons for the notorious genetic instability of strains isolated for growth on exotic substrates. There is some evidence which indicated that for certain pathways, the toluene-xylene pathway (72a) and the degradation of naphthalene (Boronin, pers. comm.) that plasmid coding appears to be the rule rather than the exception. The advantages of plasmid coding of interesting pathways, and segments of them, or both, for strain construction is almost self-evident, since they are in effect naturally cloned functions which can be readily transferred or mobilized between different hosts. Further, the gene pools are easily dissociated and re-associated between different replicons, providing a flexibility for new genetic organizations.

The Structure and Properties of the *Pseudomonas* Catabolic Plasmids

Physical characterization of these plasmids has been retarded relative to their genetic characterization for want of methods for their isolation. However, a number of methods have been developed recently which are successful. So far all the catabolic plasmids described are large relative to many R plasmids, ranging from 60 Md upwards to CAM and OCT which are in the region of 300 Md. Where incompatibility has been tested, most described are either in the IncP2 group (CAM, OCT), the IncP7 group or the IncP9 group (TOL, NAH) (8a, 61, 68). Only one physical map has so far been published and that is of the TOL plasmid, pWWO, which is 78Md (117 kb) (22). The catabolic functions of only two of these have been studied in any great detail. These are the OCT plasmid, and recombinants of it with CAM by Shapiro's group in Chicago and the TOL plasmid from *P.putida* mt-2 (referred to as pWWO in our laboratory). Because of its large size and some extremely complex endonuclease digestion patterns, the catabolic genes of OCT and its derivatives have been studied mainly by classical genetic procedures as if the genes were chromosomal: the experimental advantage of the genes being on a plasmid has been to facilitate their transfer between hosts

carrying chromosomal mutations, which has enabled unambiguous identification of some plasmid genes. Essentially only two enzymatic functions for octane utilization coded by the OCT (OCT-CAM) plasmids, an ω-hydroxylase converting the alkane to a primary alcohol and an dehydrogenase forming the corresponding aldehyde. The further metabolism is chromosomally determined and there is one or more isofunctional alcohol dehydrogenase(s) on the chromosome. Even this apparently simple array of genes are not clustered and fall into three transductional linkage groups. Details of this can be found in Shapiro's article of this volume.

The archetype TOL plasmid pWWO is much more amenable to physical investigation of its structure. Recent work has shown that it is modular in structure much as has been documented with R plasmids. Various procedures have been used to construct *in vivo* hybrids between RP4 (RP1) and TOL (14, 38, 46) and in all cases the resultant hybrid RP4::TOL appears to carry the entire R plasmid with an element of TOL incorporated into it by an event resembling transposition. The size of this transposable element varies from 56 to 65 kb in the different plasmids examined, but all of them share a common 56 kb of TOL DNA (14, 38, 46). In our laboratory we have extended this by demonstrating that in two strains of *Pseudomonas*, WR211 and PaM 1, the common 56 kb appears to have integrated into the chromosome. In one derivative of PaM 1, called PaMB 11, all of the TOL functions are expressed but there is no plasmid DNA (Williams and Wheatcroft, unpublished). From both WR211 and PaM 1 and its derivatives, the 56 kb can be rescued from its chromosomal location by mating in an R plasmid (R2, pMG 18 and RP4 have been used) and selecting for transconjugants which grow on the TOL substrates *m*-toluate or *m*-xylene. Hybrid plasmids containing the entire R plasmid with the same 56 kb of TOL DNA are formed. Hybrid R::TOL plasmids have enabled the TOL genes to be transferred out of *Pseudomonas* into *E.coli*, selecting for the resistance markers (14, 38, 46), although the catabolic genes are not as well expressed in this foreign host as in *P.putida* (38, 46, 55). It may well be that TOL itself has a broad host range for transfer but that expression is limited to a narrower range, since an antibiotic transposon bearing TOL derivative has been transferred directly from *P.putida* into *E.coli* by selecting for the resistance marker (8).

Within the transposable region of TOL, there is a section of about 39 kb which very readily deletes: this has been reported in pWWO, when a derivative pWWO-8 is formed (6), and also from an R2::TOL hybrid, from which the same DNA is deleted (Jeenes & Williams, unpub.). Loss of this region is accompanied by loss of all the TOL phenotypes and probably loss of expression of all the catabolic enzymes. The usual selection for this deletion is growth on benzoate which selects for faster growing segregants which use the alternative β-ketoadipate pathway, whereas the TOL$^+$ strains use

the plasmid pathway: total loss of plasmid and the 39 kb deletion contribute about equally in the segregants found after benzoate selection (72a).

When a transposition event is followed by a deletion event, a residual 17 kb of apparently cryptic TOL DNA is left: this has been shown to occur after benzoate selection of strains carrying the R2::TOL hybrids (Jeenes & William, unpub.). The exact location of the catabolic genes on pWWO has been investigated either by insertional inactivation using drug resistance transposons (26), by investigation of spontaneous insertion and deletion mutants or by cloning fragments into *E.coli* vectors (26, 36, Nakazawa pers. comm., Broda pers. comm.). The indications are that the catabolic genes are in two separate locations, both being on the 39 kb deletable region. The genes for the top of the pathway, responsible for conversion of the hydrocarbons (e.g. *m*-xylene) to aromatic carboxylic acids (e.g. *m*-toluate) are at one end and the genes for further metabolism of the acids are in the central region about 25 kb distant.

Hybrid Metabolic Pathways

Because of the diversity of metabolic routes available, either for the same compound in different strains or for different but related compounds in the same strain, there is a considerable potential for the construction of novel metabolic routes: these might be metabolic pathways which were rare or even non- existent in natural isolates or which did not occur in strains which are easy to handle under laboratory or industrial conditions.

One of the most fruitful areas for such pathway manipulation is in the areas of aromatic catabolism, where there are several different aerobic routes by which the aromatic ring is converted to central metabolites. The first steps of aromatic catabolism involve enzymatic conversions of the growth substrate to dihydroxyphenols, which may be derivatives of catechol (1,2-dihydroxybenzene) or quinol (1,4,-dihydroxybenzene), or both.

One of the first reports of a mutant strain which exploited the overlap of these aromatic pathways was by Feist and Hegeman (24) using *Pseudomonas putida* U. This strain grows on benzoate via catechol and the *ortho* pathway, and on phenol *via* catechol and the *meta* pathway: it can also grow on the cresols (methyl phenols) which it converts to methylcatechols and they are assimilated *via* the non-specific *meta* pathway (Figure 3). Enzymatic blockage of the *meta* pathway, followed by selection for revertants which could grow on phenol, resulted in a strain in which phenol was converted to catechol and further metabolized by the *ortho* route unlike the wild type. None of the cresols supported growth of the phenol selected

Figure 3. Pathway for the matabolism of p-substitued toluenes and benzoates with "2,3-dihydroxybenzoates" as ring-cleavage substrates. Enzymes: (1)p-Cymene hydroxylase; (2) p-isopropylbenzyl alcohol dehydrogenase (3) p-isopropybenzaldehyde dehydrogenase; (4) p-cumate 2,3-dioxygenase; (5) "dihydrodiol" dehydrogenase; (6) 2,3-dihydroxy-p-cumate 3,4-dioxygenase; (7) "ring-cleavage product" decarboxylase; (8) hydrolase; (9) hydratase; and (10) aldolase. Symbols I to X refer to the chemical intermediates in the degradative sequence XI, Pyruvate, XII, acetaldehyde, A, B, C through N refer to the proposed cistrons which encode the enzymes of the degradative sequence (18,19).

revertants as the ortho pathway is unable to totally degrade methyl catechols. These examples involve two chromosomally coded pathways. However, a very similar example involves an interaction between a chromosomal and a plasmid pathway. The interaction between TOL and the chromosomal ortho pathway which results in selection for plasmid loss or plasmid deletion has been mentioned above. In the benzoate selected derivatives, benzoate is the only one of the TOL substrates which is still utilized, as both the genes for conversion of toluene (and the xylenes) to benzoate (and the toluates) are lost, as are the meta pathway genes: the chromosomal pathway is specific and only will assimilate benzoate. However, if selection pressure for some of the TOL plasmid genes is maintained, blocked mutants can be selected. Thus it is possible to select for retention of the ability to utilize toluene but with loss of ability to grow on m-xylene. Such strains must maintain the hydrocarbon to carboxylic acid genes but utilize the chromosomal β-ketoadipate pathway, which will not degrade the methyl subtituted metabolites of m-xylene. As expected such a mutant was found to be blocked at catechol 2,3-oxygenase,

the first of the meta pathway enzymes after the divergence of the two pathways (74). This mutant therefore assimilated toluene by a novel route, namely conversion through benzoate, catechol and the ortho pathway.

A reverse example of this is with a strain P. putida S1 which grown on salicyclic acid via catechol and the ortho pathway. The first enzyme, salicylate hydroxylase which converts salicylate to catechol, is non-specific and is able to convert 3-methylsalicylate to 3-methylcatechol: it cannot grow on 3-methylsalicylate however because of the strict specificity of the ortho route for catechol only. However, by transferring TOL into P.putida S1 Nakazawa and Yokota were able to produce derivatives of S1 which could utilize 3-methylsalicylate without selection, since TOL encodes the non-specific meta cleavage pathway capable of the total metabolism of methyl catechols (47).

These examples demonstrate how it is possible to utilize knowledge of the biochemical and regulatory interactions of catabolic pathways to channel growth substrates down novel routes. Obviously formation of such hybrid pathways would be considerably easier if, in addition to the limited number of catabolic plasmids now available, there were many more and if we were able to include chromosomal DNA engineered either by in vivo techniques such as with the formation of R' plasmids or by in vitro cloning into vectors capable of replicating and expressing those genes in Pseudomonas hosts. The scope of this for converting substrates such as hydrocarbons, terpenes etc. into useful chemicals then is only limited by our extent of knowledge of the available enzymes and their specificities and the ingenuity of the molecular biologist.

It would however be wrong to assume that the sole task of an industrial microbiologist is to produce new products. Very often industrial chemists produce new compounds as wastes which are recalcitrant to biodegradation and therefore the expertise of the microbiologist is required to devise strains capable of degrading them prior to their release into the environment.

One last example of the construction of a hybrid pathway for this purpose can be quoted. Reineke and Knackmuss were interested in the problem of recalcitrance of halogenated aromatics, and isolated a strain of Pseudomonas B.13 which could degrade 3-chlorobenzoate via 3-chlorocatechol and the chlorocatechol pathway (52, 53). It has proved impossible to mutate or to select spontaneous mutants of this strain which can degrade other substituted halobenzoates. Biochemical analysis pinpointed the first enzyme, the benzoate dioxygenase, as being the block since it has a very high specificity for benzoate and 3-chlorobenzoate and will not oxidise 4-chlorobenzoate: the rest of the pathway could metabolize 4-chlorocatechol if it could be formed from 4-chlorobenzoate. To

circumvent this block, they transferred into B13 the TOL plasmid, which encodes a non-specific benzoate dioxygenase which is capable of converting 4-chlorobenzoate to 4-chlorocatechol (52, 53). Their first transconjugant WR211 is unable to utilize 4-chlorobenzoate since it is channelled into the TOL meta pathway which cannot eliminate the halogen atom (53). However, 4-chlorobenzoate$^+$ derivatives of this strain such as WR216 can be selected and they have all lost expression of the meta ring cleavage enzyme catechol 2,3 oxygenase, thus allowing the 4-chlorocatechol to be channelled into the productive chlorocatechol pathway (52). The rationale for constructing WR 216 is justified since it has been demonstrated that the benzoate dioxygenase expressed in WR 216 is the TOL specific enzyme. The adaptability of WR 216 was further demonstrated by its ability to readily form derivatives capable of growing on a dichlorinated acid, 3,5-dichlorobenzoate (52).

ENRICHMENTS FOR MUTANTS DEFECTIVE IN CATABOLISM

General methods for the isolation of mutants of pseudomonads defective in catabolic activities have usually relied on chemical mutagenesis followed by enrichment of them with penicillin selection procedures, sometimes supplemented with cycloserine (48). Two or three lytic growth cycles alternated by non-lytic growth cycles are normally required for the enrichment of the mutants to the level of 1 per cent of the population. One modification of this method allowed very high enrichments of defective catabolic mutants by providing "stress" growth conditions during a single lytic cycle (11). The details of these procedures probably require modification for each strain of pseudomonad isolated for its specific catabolic activity of interest. The modifications are likely to extend to the nature and quantity of the mutagen used, as well as to the effectiveness of the antibiotics prescribed in the lytic cycles. As is well known when mutagens are used to obtain desired mutants it is desirable that a single locus has been affected with reasonable certainty, and this is usually ensured by the transfer of the mutant allele to a known parental genetic background. In general this has not been possible in pseudomonads, at least for putative chromosomal genes. For catabolic genetic lesions located on transmissible plasmids, this is not a problem in the strains intensively studied.

Direct and specific enrichments of catabolic mutants in bacteria have been achieved by two means which require, either the use of a genetically modified host, or exposure to a substrate analogue. In the first situation, a metabolic lesion in a nonessential pathway may allow the intracellular accumulation of a normal metabolite to concentrations which are toxic to cells in which it is formed. In the alternative condition a substrate

Figure 4. Pathways used for the catabolism of phenol, cresols. benozate and m-toluate by P. putida U.

analogue is partially metabolized to an analogue metabolite which is toxic. The latter approach was used by Apirion in 1962 (3) with a fluorinated substrate analogue for the enrichment of both auxotrophs and prototrophs in Aspergillus sp. Fluoroacetamide was used as a substrate analogue to obtain mutants of P. aeruginosa defective in acetamide catabolism (17). The success of this approach is thought to be due to the conversion of fluoroacetamide to a metabolite analogue such as fluorocitrate, a potent inhibitor of aconitase (but see 10). The enzyme-catalyzed formation of fluorocitrate from fluoroacetate was called a lethal synthesis since it caused respiration to cease (51).

Substrate analogues containing other halogens have not been used for similar purposes, although some biochemically well defined catabolic pathways seem suitable for the rational choice of halogenated analogue substrates that could give rise to toxic analogue metabolites. This opportunity has been explored with the pathway for p-cymene metabolism in P. putida (69). The enzymes of the p-cymene pathway (Figure 3) possess a relaxed specificity such that

TABLE III
Enrichment of P. putida Mutants Defective in p-Cymene Degradation after Exposure to Halogenated Analogues*

Analogue	No. of defective mutants/ no. of total survivors tested
α - Chloro-p-xylene	18/125
p - Chlorotoluene	39/125
p - Bromotulene	9/76
p - Iodobenzoate	69/76
p - Fluorobenzaldehyde	0/76

* Taken from Wigmore and Ribbons (1980).

several substrate analogues are transformed to products in defective mutants (18,19) including halogenated analogues (69). Some halogenated analogues of p-cumate might be expected to be precursors of "halo-catechols" or acyl halides and halo-methyl ketones should they be substrates for ring cleavage. It has not been determined whether or not these analogue intermediates are formed in P. putida, however, some chloro-bromo- and iodo-analogues of p-cymene are useful for the enrichment of mutants defective in the pathway, without known mutagenesis (69). Table III shows an example of the extent of the enrichments achieved after three days exposure of cells to the analogues.

Exposure of cell suspensions to appropriate halogenated substrate analogues has provided significant enrichments of defective mutants in seven other catabolic pathways (70) (Table III). The procedure has also provided up to six different defective phenotypes from a single enrichment in the phenol-cresol pathways in P. putida (70). However incubation of cells harboring TOL functions with 3-fluoro-p-toluate, like benzoate, gives rise to non-revertable TOL^- strains.

VECTORS FOR CLONING IN PSEUDOMONADS

All vectors so far described for cloning genes into pseudomonas are plasmids; bacteriophage genomes such as λ (double-stranded DNA) or M13 (single-stranded DNA), as used in E. coli have not been developed for pseudomonads. Cloning vectors for pseudomonads have been constructed from: (i) medium-sized (50-60kb) conjugative plasmids of the P-1 incompatibility group, e.g. RP1 (RP4, RK2, R68) and W-incompatibility group e.g. S-a and (ii) small (9kb) non-conjugative plasmids of the Q incompatibility group, e.g., RSF1010

TABLE IV
Substrate/Substrate Analogue Pairs Used for Enrichment of Mutants Defective in Catabolism*

Strain	Substrate/analogue	% Mutants in population
P.putida U	p-Toluate/3-fluoro-p-toluate	55
P.putida AC-2	Salicylate/5-fluorosalicylate	11
P.putida PpG1064	Salicylate/5-fluorosalicylate	19
P.fluorescens JT701	4-Methylphthalate/4-chlorophthalate	30
P.putida U	4-Hydroxybenzoate/3-fluoro-4-hydroxybenz-oate	22
P.putida JT101	p-Anisate/3-fluoro-p-anisate	96
P.putida U	Phenol/3-fluorophenol	97
P.putida U	Phenol/3-chlorophenol	98

* Taken from Wigmore and Ribbons (1981)

(R1162,R300B). Both Inc P-1 and Inc Q (P-4) plasmids are promiscuous in that they can be transferred to a wide variety of Gram-negative bacteria and stably replicate. The former group suffer from their large size and low copy number, whereas the latter group are more representative of the most useful high copy number vectors developed for E. coli.

Vectors Derived from Large Inc P-1 Plasmids

A non-transmissible variant of RP4 was proposed as a suitable broad host range cloning vehicle in 1975 (37). Several other derivatives of RP4 have been constructed which possess unique sites for Bam HI, EcoRI, Hind III and SalI (5). Ditta et al., (21) have used a smaller non-conjugative derivative of RK2 with useful Bgl II and EcoRI sites for the construction of a gene bank in Rhizobium; its usefulness as a vector in Pseudomonas spp. is not known, however for information on other derivatives of RK2 available for cloning Helinski's plenary communication of this volume should be consulted. De Wilde et al. (20) have made a broad host range plasmid, pGV1106, from the S-a plasmid which has unique cleavage sites for the endonucleases Bgl II, Bam HI, EcoRI and Kpn I.

Vectors Derived from Small Multi-copy Inc Q (P-4) Plasmids

Some Inc Q plasmids (RSF1010, R300B) isolated from enterobacters

are thought to be identical to the Inc P-4 plasmid R1162 found in P. aeruginosa (61). They are about 9kb in size, code for streptomycin and sulphonamide resistance, multi-copy (15-20) and mobilizable by conjugative plasmids.

Timmis's group has modified plasmid RSF1010 in a number of ways to provide suitable cloning vectors for endoculeases which do not cleave the parent plasmid (4, Timmis pers. comm.). pKT210 is a combination of the large fragment of RSF1010 and a fragment from plasmid S-2 carrying the chloramphenical (Cm) resistance gene, both generated by Pst II cleavage. This Cm gene provides a unique Hind III cleavage site for the recombinant pKT210 plasmid. Hind III fragments cloned into pKT210 are recognized by insertional inactivation of the Cm gene but retention of Sm resistance. The opposite resistance pattern is shown for fragments cloned in the Eco RI (Hpa I) nd Sst I and Bst EII sites.

Vector pKT 248 contains another Cm resistance fragment (from R621a1a) into a Pst II site of RSF1010 and this provides a Sal I site for recognition of cloned sequences by inactivation of Cm resistance. By insertion of a Pst II fragment within a Kanamycin (Km) resistance gene into RSF1010 to give pKT 105 cloning sites into Hind III, Xma I, Xho I cleavages are generated. Other examples of vectors for Cla I, Bam HI, Hind III, Sal I are derived by combining a second plasmid to the P-4 plasmids, e.g. pKT230 and pFG7, the resultant vectors having two different origins for replication (Table V).

Cosmid Vectors for Pseudomonads

Cosmids are plasmid vectors containing a region of phage DNA with a specific recognition sequence (cos) that is essential for packaging of recombinant molecules into the head proteins to give a mature virus. The advantage of this type of vector is that there is a strong selection for the cloning of long DNA fragments of large (≃40 kb) and of reasonably uniform size. This is due to the requirement of mature phages to package a piece of DNA near to the phage genome size. The only cosmids so far constructed use the cos sequence of phage λ of E. coli. Since phage λ does not infect nor replicate in pseudomonads the cloning of Pseudomonas genes needs to be done in E. coli followed by a second stage transfer of recombinant cosmid DNA by transformation to Pseudomonas. The cos sequence of λ DNA has been cloned into P-4 plasmids to give cosmids and these may be used for the cloning of DNA fragments derived from a variety of endonucleases (Table V).

Gene Cloning in Pseudomonas

To date there have been very few reports of cloning of Pseudomonas catabolic genes. The most detailed report to date has

TABLE V
Some Vector Plasmids for Cloning in Pseudomonas

Vector	Replicon(s)	Size (Kb)	Cloning sites
pKT210	RSF1010	11.8	EcoRI, SstI, Hind III
pKT248	RSF1010	12.4	EcoRI, SstI, Sal I
pKT230	RSF1010 pACYC177	11.9	EcoRI, SstI, Hind III XhoI, XmaI, BstEII BamHI
pKT247*	RSF1010	11.5	EcoRI, SstI
pFG6*,a	R1162 pHC79	15.3	ClaI, BamHI, Hind III SalI

Data from Bagdasarian and Timmis, personal communication.

* Cosmids

[a] Gautier, F. and R. Bonewald. Molec.Gen.Genet. 178: 375-380 (1980).

been the cloning of part of the P. aeruginosa aliphatic amidase genes in a bacterophage λ vector; the cloned fragment contains the entire structural gene amiE and part of the gene for the positive regulator amiR. The TOL plasmid and parts of it have also been cloned in three laboratories (4,26,36,Nakazawa, pers. comm., Broda, pers. comm.).

However, if expression of cloned Pseudomonas catabolic genes is required then it is probably necessary to have vectors which themselves replicate well in Pseudomonas. On the very limited evidence available it seems that Pseudomonas biosynthetic genes can express and restore prototrophy in intergeneric crosses into E. coli (27,28,45), but catabolic genes express too poorly to sustain growth (8,38,46,55). The latter may only a a temporary problem and it may prove possible to select for mutants which acquire higher expression. However, to make use of the full diversity of catabolic genes in the genus, specific vectors for Pseudomonas seem essential.

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BEGINNING GENETICS WITH METHANOGENS

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"So methanogens no longer are second-class citizens of the microbial world." R. S. Wolfe (1979)

There have been two meetings*, convened during the past year, specifically to determine how microbial genetics could be productively applied to obligately anaerobic microorganisms and to methanogenic species in particular. One of us (JNR) attended both of these meetings. The following account of the status of current knowledge of the molecular biology of methanogens and suggestions for genetic approaches to be used in their study are, in part, derived from the very open and constructive discussions held at these two meetings.

WHY THE "SUDDEN" INTEREST IN METHANOGENS?

For Dr. Wolfe (see opening quotation) and a few other investigators, there is no "sudden" interest. They have investigated methane producing organisms for many years and have surmounted enormous technical problems to produce an impressive body of knowledge describing the taxonomy, fine structure, physiology, and growth requirements of many methanogens and have begun detailed biochemical analyses of the intermediary metabolism leading to

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methane production. This information can be found in several recent reviews (2,21,29,30). The "sudden" interest of molecular biologists seems, however, to be based on three factors. First, relatively simple techniques have been developed for handling methanogens and for obtaining growth in liquid or on solid media using chemically defined media. Second, methanogens belong to a group of organisms collectively named Archaebacteria (3,28) which do not conform with classical taxonomy and which appear to be neither truely procaryotic nor eucaryotic. Studying the molecular biology of these organisms will therefore undoubtedly provide novel information and, in particular, should help define which processes/structures are common to all biological systems and which can be modified or replaced with alternative mechanisms. Third, the advent of recombinant DNA technology has opened the possibility of studying the expression, and regulation of expression, of methanogen derived DNA in such "standard" microorganisms as Escherichia coli, Bacillus subtilis, and Saccharomyces cerevisiae. Even though the possibility of using recombinant DNA techniques to produce methanogens with increased growth rates, or higher methane yields or to transfer the ability to produce methane to other species currently appears remote, it cannot be ignored. The enormous industrial and governmental investments in both recombinant DNA research and the search for renewable energy sources cannot but ensure that recombinant DNA technology will be focused on and used to evaluate the potential of methanogenic species.

DNA STRUCTURE IN METHANOGENS

The term methanogen encompasses a group of morphologically and physiologically diverse organisms with DNA contents ranging from 27.5 to 52 mol% G+C (2). This range is almost as broad as the range exhibited by all procaryotes. Only one report is available describing the DNA content and structure of the genome of a particular methanogen (19). Mitchell et al. (1979) showed that the genome of Methanobacterium thermoautotrophicum has a complexity of 1.1×10^9 daltons; approximately 40% the size of the genome of E. coli. Their results also indicated that approximately 6% of the DNA from M. thermoautotrophicum can renature very rapidly, suggesting the presence of repetitive or "fold-back" DNA. It is clearly very important to determine if a small sized genome is a common characteristic of methanogens or whether the species chosen by Mitchell, et al. (1979) is not representative of methanogens in general. It is also of basic importance to establish whether extra-chromosomal elements exist in methanogens. One preliminary report indicates the presence of small circular DNA molecules in two methanogenic species (Howard, G. and J. Frea. Bact. Proc., 1980, p. 113), however, these molecules do not seem to exhibit the physical characteristics, such as supercoiled structure, normally associated with plasmids (G. Howard, pers. com., 1979).

RNA SYNTHESIS AND STRUCTURE IN METHANOGENS

DNA-dependent RNA polymerase is considered by many molecular biologists as the pivotal enzyme in gene expression and as such has been purified from many sources and studied in depth. Recently results of such studies with enzymes isolated from Archaebacteria hve been published (27,31). The enzymes from these organisms, including Methanobacterium thermoautotrophicum, do not conform to the procaryotic subunit configuration $(\beta'\beta\alpha_2)\sigma$ but exhibit a totally different composition. The active enzyme isolated from M. thermoautotrophicum has two forms; I, subunits with molecular weight 98,000; 73,000; 59,000; 52,000; and 22,000; and II, subunits with molecular weights 73,000; 59,000; 38,000; 24,000; 18,000; and 13,000. This enzyme is oxygen sensitive and is resistant to the inhibitors of procaryotic RNA polymerases, rifampin and streptolydigin and to α- amanitin which inhibits the B and C types of eucaryotic RNA polymerases. In contrast to all procaryotic RNA polyerases studied, the enzyme from M. thermoautotrophicum does not specifically recognize the early promoters of phage T7 DNA and appears to bind to T7 DNA in a manner totally unrelated to the known promoter locations on T7 DNA (K.O. Stetter; pers. comm. 1981). Although all DNA-dependent RNA-polymerases isolated from Archaebacterial species so far do not have the $(\beta'\beta\alpha 2)\sigma$ subunit sructure and do show some common features in their subunit structures and drug resistances, it is also certain that these enzymes are very diverse. No antigenic relationship can be shown between the DNA-dependent RNA-polymerases isolated from methanogenic species and the enzymes isolated from other Archebacterial groups (27). Fundamental differences between the structure and probably promoter recognition properties of methanogen-derived RNA polymerases as compared to the analogous enzyme in E. coli obviously pose both a challenge to the biochemist to determine how such radically different enzymes can catalyze the same reaction and a potential problem for recombinant DNA studies.

The structure of mRNA molecules in methanogens is currently unknown. In contrast, the structures of tRNAs and rRNAs from several methanogens have been analyzed in depth (2,6,10). It was essentially on the basis of comparisons of oligonucleotides resulting from nuclease digestions of rRNAs that Woese et al. (28) proposed that a group of organisms, which includes methanogens, be separated from both procaryotes and eucaryotes and be designated 'Archaebacteria'. The oligonucleotides derived from the 16S rRNA of methanogenic species are very different from both the procaryotic and eucaryotic equivalents. One potentially very important similarity was, however, pointed out by Steitz (25). The region of the 16S rRNA molecule thought to be involved in RNA"RNA duplex formation which directs the binding of mRNAs with rRNA as required for initiation of protein synthesis is highly conserved in all procaryotic species and is also present in the sequence of 10 rRNAs isolated from methanogens.

This suggests that mRNAs from methanogens may, in fact, be able to correctly interact with ribosomes inside procaryotic cells. Analyses of the structure of tRNAs from methanogens have shown that these molecules also do not closely resemble the tRNAs of either procaryotes or eucaryotes (6,10). Differences in unusual base composition and in the types and extent of post-transcriptional modifications of tRNA have been catalogued. The overall biological role of these modifications is currently unclear and therefore the functional significance of the differences between the structure of tRNAs from methanogens and from other sources cannot presently be evaluated.

PROTEIN SYNTHESIS IN METHANOGENS

Although ribosome function is conserved in procaryotes and eucaryotes, there are major structural differences in ribosomes from these two biological kingdoms. The ribosomes from methanogenic species appear, at least superficially, to be of the procaryotic type (8). The structures of ribosomes from several methanogens have been studied and they conform to the 30S plus 50S subunit configuration. The 30S subunit has approximately 21 ribosomal proteins and the 50S subunit has approximately 31 ribosomal proteins. Two-dimensional gel electrophoretic separations of these ribosomal proteins do, however, demonstrate that ribosomal proteins from methanogens are significantly more "acidic" than are their classical procaryotic counterparts (8). More detailed studies suggest that ribosomes and individual ribosomal proteins are, in fact, very different from their procaryotic equivalents. Antisera raised against ribosomal proteins of *Methanobacterium bryantii* do not cross react with ribosomes or ribosomal proteins from procaryotes or from yeast (24). The primary sequence of ribosomal protein 'A' from *Methanobacterium thermoautotrophicum* (equivalent to the *E. coli* ribosomal protein L7/L12) is very different from the structure common to many procaryotes and more closely resembles the 'A' sequence from cytoplasmic components of eucaryotes (18). Evidence also suggests that the ribosome associated elongation factor (EF-Tu) present in *Methanococcus vannielii* is also not structurally related to other procaryotic elongation factors (9). The observation that diphtheria toxin can ADP-ribosylate an elongation factor from *Methanobacterium thermoautotrophicum* presents convincing evidence that the components of the protein synthesizing machinery in this methanogen is very different from its procaryotic equivalent and, indeed, indicates a closer relationship with its eucaryotic equivalent (17).

Development of an *in vitro* protein synthesizing system derived from *M. barkeri* is a most important development (A. Böck, pers. comm. 1981). This system can use polyribouridylic acid to direct the synthesis of polyphenylalanine. Böck has shown that polypeptide biosynthesis in this *in vitro* system is not inhibited by chloramphenicol and that radioactively labeled chloramphenicol does not

bind to ribosomes from methanogens. This is an important observation as chloramphenicol does inhibit the growth of methanogens and must, therefore, do so by a mechanism other than inhibition of protein synthesis (11,20). Analysis of the products of *in vitro* synthesis, using this system primed with natural mRNAs, e.g. MS2, should provide very important insights into the macromolecular synthetic machinery of methanogens. In a related study, Böck (pers. comm., 1981) has been unable to detect a stringent response in methanogens, i.e. RNA synthesis continues *in vivo* even if protein synthesis is inhibited. There is no detectable accumulation of guanosine-5'-diphosphate-3'-diphosphate (ppGpp).

MACROMOLECULES OF THE CELL ENVELOPE

The cell walls of methanogens do not contain muramic acid or D-amino-acids but contain analagous sugar-peptide polymers which presumably play an equivalent structural role to the bacterial peptidoglycan. In addition, the lipids found in methanogens contain ether linkages in contrast to the ester linkages found in lipids from other sources (2,28). It is most important to recognize these differences as many antibiotics used extensively as selective agents in bacterial genetics are active because they inhibit a stage in the synthesis of the bacterial cell envelope. These antibiotics are, to a large extent, inactive against methanogens and therefore cannot be used as selective agents in genetic experimentation with methanogens (11,20).

DEVELOPMENT OF A GENETIC EXCHANGE MECHANISM

To develop a genetic exchange system one needs selectable traits to screen and identify recombinants. As it is now possible to grow methanogens as colonies on solid media, it should be possible to isolate drug-resistant mutants, auxotrophic mutants and temperature-sensitive mutants. As noted above, the majority of antibiotics used extensively in bacterial genetics do not inhibit the growth of methanogens. There are, however, a few drugs, e.g. bacitracin, gramicidin S, monensin which are active against methanogens and which could be used to select resistant mutants (11). Isolation of auxotrophs should also be possible although it may involve a large amount of labor as penicillin-enrichment schemes cannot be used. Isolation of auxotrophs may require very high concentrations of media supplements as active transport of amino-acids, other than cysteine, has not been observed in methanogens. Uptake of intermediary metabolites from the external environment has only been detected in the presence of large external concentrations of the metabolites. (R. Thauer, pers. comm., 1981). Although methanogens may not incorporate amino acids, they do excrete amino acids, mostly L-alanine (R. Thauer, pers. comm., 1981) and it may be possible to

isolate mutants defective in this process by their inability to cross-feed amino-acid auxotrophs of other species. While auxotrophic mutants in general would be of value, it would be extremely useful to obtain mutants in which the biochemistry of methane production had been altered. The central cofactor, unique to methanogenic species, involved in methane biosynthesis is called coenzyme M and has the structure $HS\text{-}SCH_2CH_2SO_3^-$ (2,4). One species, Methanobacterium ruminantium M1 is naturally auxotrophic for this compound and can therefore be used to assay the effectiveness of analogs of coenzyme M and the inhibitory effects of competitive inhibitors of coenzyme M (4). One inhibitor, bromoethanesulphonic acid, effectively blocks growth and methane production. Isolation of mutants resistant to this and similar compounds should provide a means of obtaining mutations which effect the enzymes and cofactors directly involved in methane biosynthesis. A recent report that iodopropane also blocks both growth and methane production suggests that this compound could also be used as a selective agent (16).

The availability of mutants is only the initial step in developing a genetic exchange system. A subsequent major problem will be to successfully transfer, and to recognize the transfer of, DNA from the donor strain to the recipient. Natural systems of DNA transfer in bacteria range from the uptake of naked DNA via transformation to complex systems mediated by sexual proficient plasmids and by transducing phages. Plasmids and phages are currently unknown in methanogens and although experience strongly suggests that they exist and will be found if sought correctly, it is most likely that a DNA mediated transformation system or cell:cell fusion system will most readily be devised. A wide variety of regimes has been established for obtaining bacterial cells competent for the uptake of DNA and it is impossible to predict which, if any, of these techniques will facilitate transformation of methanogens. Two points should be recognized in designing potential transformation/cell fusion protocols. First, there are currently no enzymes known equivalent to lysozyme which could be used to digest or weaken the cell envelope of methanogens. Second, Methanococcus vannielii may be an astute choice as the experimental system as this species, unlike other methanogens, is easy to lyse (13,14) for obtaining high molecular weight DNA or for rapid screening for the presence of plasmids (when available) and should form protoplasts if handled correctly (R. Wolfe, pers. comm., 1981).

ANALYSES OF METHANOGEN DERIVED DNA USING RECOMBINANT DNA TECHNOLOGY

Many of the technical problems associated with the direct genetic analysis of methanogens may be circumvented by analyzing DNA derived from methanogens in a surrogate host. We have constructed recombinant DNA molecules by ligation of DNA derived from Methanosarcina barkeri to two different cloning vectors. The vectors

chosen, λ1059 (15) and cosmid pHC79 (12), were designed to permit the cloning of very large fragments of DNA. This, in turn, dramatically reduces the number of independent recombinant clones needed to obtain a gene bank (7). If one assumes that the genome size of M. barkeri is the same as that of Methanobacterium thermoautotrophicum (19) and that an average size of 18 Mdal is selected for the cloned methanogen DNA then a gene bank as defined by Clarke and Carbon (7) will comprise less than 200 independent clones. Large numbers of recombinant clones have been constructed by incorporating BamHl or BclI generated restriction fragments of M. barkeri DNA into λ1059 and by incorporating HindIII generated fragments into pHC79.

ANALYSIS OF λ1059-M. BARKERI CLONES

Figure 1 shows examples of the recombinant DNA molecules which are formed by ligation of the lambda vector phage with BamHl restricted M. barkeri DNA. We have used phage infection of minicells to determine if the M. barkeri DNA, cloned in λ1059, can be expressed in E. coli (23). Minicells do not contain DNA and therefore infection results in the synthesis of phage encoded polypeptides in the total absence of synthesis of E. coli proteins. Infection of minicells with lambda phages carying cloned or transduced DNA results in the synthesis of both lambda polypeptides and polypeptides encoded by the non-lambda DNA. If minicells are used which contain a high level of lambda repressor protein then transcription can only initiate at promoters insensitive to this repressor, i.e., promoters carried by the cloned or transduced DNA (22). Polypeptides synthesized in minicells infected with several λ1059-M. barkeri clones, in the presence and absence of lambda repressor, are shown in Figure 2. Polypeptides synthesized in all infections in the absence of lambda repressor are lambda gene products. In addition to the lambda gene products, there are polypeptides whose synthesis is dependent on the individual recombinant phage being used. These polypeptides are presumably encoded by the cloned M. barkeri DNA. Some may be polypeptides encoded by the DNA at the junction of the lambda and M. barkeri DNAs and, as such, would be neither authentic lambda nor M. barkeri polypeptides but would be "fusion" polypeptides. Comparisons of the polypeptides synthesized in the presence and in the absence of lambda repressor clearly indicates that the majority of the polypeptides are translated from mRNAs which are initiated at lambda promoters. Some of these polypeptide (e.g., the polypeptide designed 'A' encoded by λ1059-M. barkeri 505; Figure 2) are, at least, partially encoded by M. barkeri DNA. More surprisingly there are also polypeptides (B,C,D,E, and F encoded by λ1059-M. barkeri 506 and 510; Figure 2) which must be translated from mRNAs which are transcribed from promoters insensitive to lambda repressor. This observation indicates that there are sequences contained in M. barkeri DNA which can act as promoters for E. coli RNA polymerase. It does not, of course, mean that these sequences

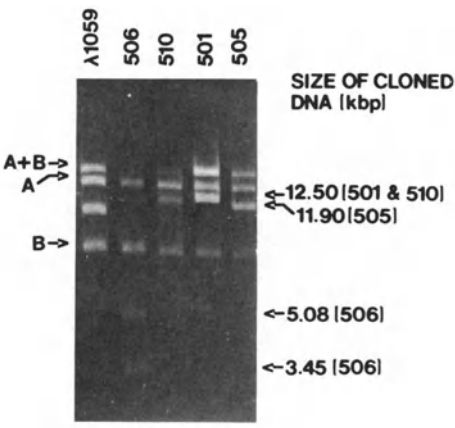

Figure 1. Electrophoretic separation of the products of BamHl restriction endonuclease digestion of λ1059-Methanosarcina barkeri DNAs. BamHl (Miles Lab. Elkhart Ind.) was used to digest DNA isolated from M. barkeri and from λ1059 (4 units enzyme/μg DNA) in 10 mM Tris HCl ph7.5; 10 mM $MgCl_2$; 50mM NaCl for 30 min at 37°. 3 μg of digested M. barkeri DNA was mixed with 1 μg digested vector DNA and incubated overnight at 15° in the above buffer containing 0.1 mM ATP; 10 mM β-mercaptoethanol and 7 units of T4 DNA ligase (New England Nuclear, Boston, Mass.). Following ligation, the reaction mixture was added to an in vitro packaging mix (26) supplemented with partially purified λ'A' protein (5) kindly donated by A. Becker. The mixture was subsequently used to infect E. coli Q359 (hsr- hsm+ syII+ϕ80RP2). The vector cannot grow on this strain (15) whereas recombinants in which the central BamHl fragment has been replaced with M. barkeri DNA can grow provided that the cloned fragment of DNA falls within the size range of 6-24 kb (15). Plaques formed by the growth of λ1059-M. barkeri recombinant phages were picked. Stocks of individual clones were grown from which DNA was isolated. The isolated DNAs were digested with BamHl, the resulting DNA fragments were separated by electrophoresis through 0.6% agarose gels and visualized by irradiation at 330 nm following ethidium bromide staining. The figure above each track represents the designation given to different clones. The vector is at the left. The restriction fragments A and B, common to all phages, are the left and right arms of the vector phage and A + B is the combination of these fragments hybridized together via their cohesive ends. Phages 510, 501, and 505 contain a single BamHl generated fragment of M. barkeri BNA whereas 506 contains two fragments.

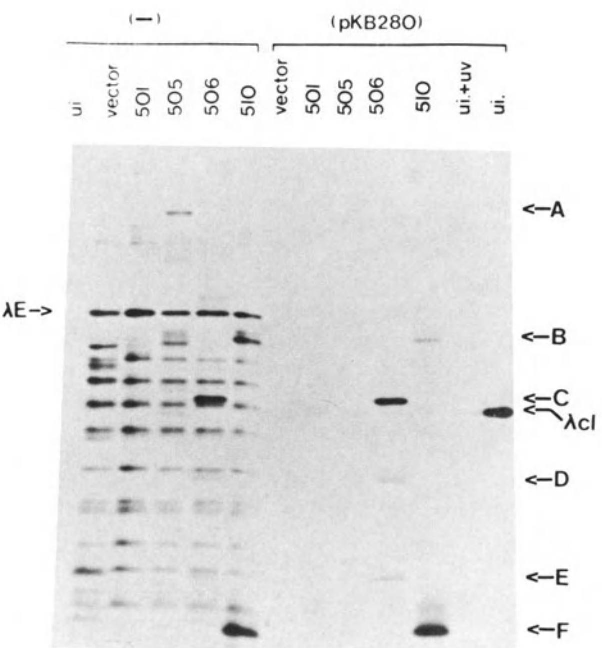

Figure 2. Autoradiogram of the electrophoretic separation of ^{35}S-methionine labeled polypeptides synthesized in minicells infected with λ1059-M. barkeri recombinant phages. Minicells prepared from E. coli DS410 (23) and E. coli DS410 (pKB280) (22) were infected with the phages designated above each track (see Figure 1 for DNA structure). Infected minicells were allowed to incorporate ^{35}S-methionine for 1 hr at 37° before being lysed and the lysate analyzed by electrophoresis through a 10-20% polyacrylamide gradient gel followed by fluorography. Techniques and references to techniques used to analyze phage products by infection of minicells have been published (22,23).

Plasmid pKB280 directs the synthesis of large amounts of lambda repressor protein (cI) (1) and minicells containing this plasmid must be U.V. irradiated before infection to destroy the template activity of the plasmid. Synthesis of λcI (Mr=26,000) in unirradiated, uninfected minicells produced by E. coli DS410 (pKB280) is shown in the right hand track. The effects of irradiation are seen in the adjacent tracks. Polypeptides presumed to be encoded by methanogen derived DNA are indicated (A-G) to the right of the figure. The major lambda capsid protein, product of the lambda cistron E (Mr=38,000) is indicated to the left of the figure.

represent promoters recognized by M. barkeri DNA polymerase in M. barkeri cells. Nevertheless, it demonstrates that mRNA transcribed from M. barkeri DNA can be recognized by ribosomes in E. coli and that tranlation of this mRNA to produce relatively large polypeptides (B has a molecular weight of approximately 36,000) can occur in E. coli minicells.

ANALYSIS OF pHC79-M. BARKERI CLONES

The cosmid vector pHC79 carries drug resistance determinants for ampicillin and for tetracycline. We cloned HindIII fragments of M. barkeri DNA into pHC79 such that the encoded ampicillin resistance was unaffected but tetracycline resistance was destroyed. M. barkeri DNA was only partially digested so that the recombinant molecules construced were sufficiently large to permit their in vitro packaging into infectious lambda particles (26). These particles were used to infect E. coli strain 5K (thr, thi, hsr$^-$, hsm$^+$) (7). E. coli 5K does not contain the K12 restriction endonuclease but does contain the K12 modification system such that the infecting recombinant DNA molecules were not subject to restriction and became modified during subsequent replication. Ampicillin resistant clones, in which the recombinant molecules had established as plasmids, were selected from the infected culture. A large number of clones were mixed and infected with λb221 cI26. Infection produces a lysate which contains both λb221 cI26 particles and lambda particles carrying the repackaged recombinant cosmids. These lysates were used to infect E. coli χ760 ara-1 leu-1 azi^R tonA^R lacY2 proC119 tsx purE1 galK2 trp3 his4 argG rpsL xyl-1 mnl-1 ilvA6 thi1 met12 which carried the excision defective prophage λxis$^-$ red3 c1857. Ampicillin resistant transductants were selected and screened for their ability to grow in the absence of one of the amino acid requirements of E. coli χ760. Four ampicillin-resistant clones (from a total of 200 tested) were found to no longer require arginine for growth. Suppression of the other auxotrophic requirements of χ760 was not detected. Further analyses of the ampicillin resistant, arginine independent clones have demonstrated the presence of recombinant plasmids carrying the cosmid pHC79 sequence ligated to M. barkeri DNA. These observations indicated that E. coli can, in fact, transcribe M. barkeri DNA and translate the resulting mRNA to produce a functional enzyme in E. coli. If the interpretation of this result is correct it would indicate that the genetic code of M. barkeri is the same as that of E. coli and at least this gene product is not encoded by a gene carrying an intervening sequence. Further details of these experiments will be presented elsewhere. (Hamilton and Reeve, in preparation).

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CHLOROPHYLL-BINDING PROTEINS: STRATEGIES AND DEVELOPMENTS FOR DNA CLONING IN *RHODOPSEUDOMONAS SPHAEROIDES*

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INTRODUCTION

The three bacterial representatives capable of anoxygenic photosynthesis are members of the family *Rhodospirillaceae*, *Chromatiaceae* and *Chlorobiaceae* (10). Although there are many physiological and anatomical distinctions characterizing each of the families, a major distinction is that the former two families have both their light-harvesting (LH) and reaction-center (RC) activities within the same membrane system (9), whereas members of the *Chlorobiaceae* have structurally separated these activities (3). Many representatives of the *Rhodospirillaceae* in addition to growing photoheterotrophically are capable of chemotrophic growth. On the other hand, the *Chromatiaceae* and *Chlorobiaceae* are, by and large photoautotrophs (9). Generally, these organisms require a simple salts medium supplemented with a few common B-vitamins. Finally, the purple bacteria, *Rhodospirillaceae* and *Chromatiaceae* contain either bacteriochlorophylls (Bchl) *a* or *b* (11) (some Bchl*a* containing species have 5% Bchl*b*:6), while members of the *Chlorobiaceae* contain in addition to Bchl*a*, Bchl's *c*, *d* or *e* (7).

The minimal functional requirements associated with photosynthetic growth of these bacterial groups involve the entrapment of incident light by light-harvesting pigment-protein complexes and the transfer of the electric excitation to the reaction center where primary charge separation occurs (12).

Although there are many diverse genera and species of photosynthetic bacteria occupying a variety of environmental niches only a handful have been intensively studied in the laloratory. In our

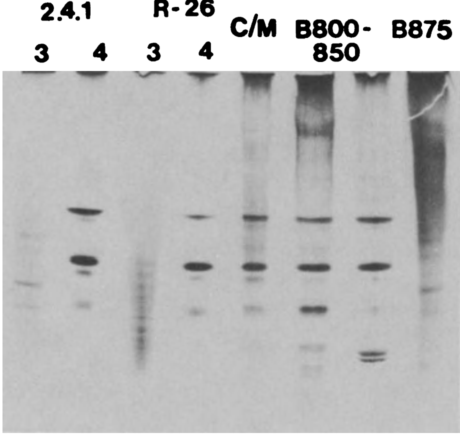

Figure 1. Isoelectric focusing of light-harvesting polypeptides. Under 2.4.1: 3-B875 fraction solubilized without detergents, 4-B800-850 fraction solubilized without detergents; Under R-26: 3-B875 fraction solubilized without detergents and missing B875 polypeptide, 4-B800-850 fraction solubilized without detergents; C/M, chloroform/ methanol soluble B800-850 polypeptides; Under B800-850: two different preparations of spectrally active detergent solubilized complex; B875, preparation of spectrally active detergent solubilized complex.

laboratory we have been most concerned with members of the Rhodospirillaceae. Rhodopseudomonas sphaeroides and similar facultative photosynthetic bacteria represent the most physiologically diverse group of organisms known.

General Description of Rhodopseudomonas sphaeroides Physiology

Rps. sphaeroides is a gram negative microorganism able to grow chemotrophically in the presence of oxygen whereby it oxidizes a variety of organic acids to CO_2 and H_2O employing an electron transport chain, similar to that found in the mitochondrion and using oxygen as the terminal electron acceptor (9). Anatomically it is similar to most other gram negative bacteria. Upon the removal of oxygen a series of events is triggered which for the most part are only partially defined, and which result in the differentiation of the cytoplasmic membrane through a process of invagination to two membranous domains (8). The new domain represents the intracellular localization of the light-dependent photosynthetic activities. Although the removal of oxygen triggers photosynthetic membrane (PM) development, the actual amount of PM formed is inversely proportional to the incident light intensity (8)and can vary from 10-15% to as much as 30-40% of the cellular dry weight. These organisms utilize light energies in the near-infra-red region of the spectrum (750nm -

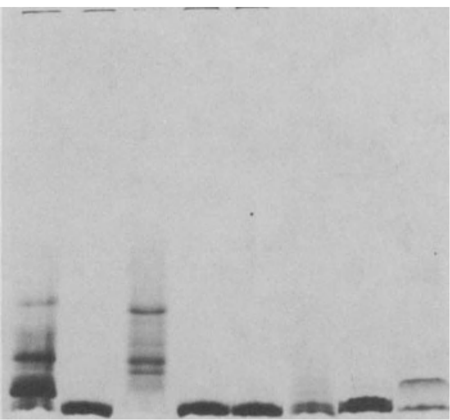

Figure 2. SDS-PAGE of light-harvesting polypeptides. Under 2.4.1: 3-B875 fraction solubilized without detergents, 4-B00-850 fraction solubilized without detergents; Under R-26: 3-875 fraction solubilized without detergents and missing B875 polypeptide, 4-B800-850 fraction solubilized without detergents; C/M, chloroform/methanol soluble B800-850 polypeptides; Under B800-850: two different preparations of spectrally active detergent solubiized complex; B875, preparation of spectrally active detergent solubilized complex.

950nm)(15), which are largely unavailable to other living systems. In addition to the utilization of light quanta these organisms will synthesize ribulose-1,5-bisphosphate carboxylase in order to fix CO_2 (5). Depending upon the external electron source this enzyme can represent as much as 30% of the soluble cell protein. Depending upon the genus and species, the structure of the enzyme can be a one subunit-type enzyme or a two-subunit type enzyme.

Furthermore, under phototrophic conditions and in the absence of assimilatable nitrogen, Rps sphaeroides can fix N_2 gas (18). Additionally, these organisms are weakly fermentative (16). In the absence of light under anaerobic conditions they can utilize an exogenous electron acceptor employing an anaerobic respiratory form of growth. One very good acceptor is dimethylsulfoxide (17). Finally, these organisms can be grown phototrophically on H_2 and CO_2, or they can be grown under conditions which lead to the liberation of large quantities of H_2 (18).

Because so much is known regarding the capture of light energy, charge separation, and photosynthetic electron transport in Rps.

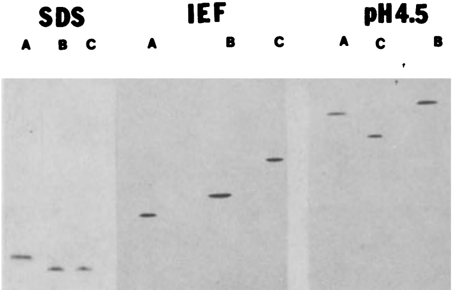

Figure 3. Purified light-harvesting polypeptides following the non-detergent solubilization procedure (1), under SDS: SDS-PAGE of A which is B875 polypeptide, B and C which are B800-850 polypeptides; under IEF: IEF of A which is B875 polypeptide, B and C which are B800-850 polypeptides; under pH 4.5: pH 4.5 gel electrophoresis of A which is B875 polypeptide, B and C which are B800-850 polypeptides.

sphaeroides we have centered our studies on an understanding of the physiology, genetics and molecular biology of photosynthetic membrane development. Although time precludes a discussion of much of our work one facet of these studies is of special relevance here.

BACTERIOCHLOROPHYLL-BINDING PROTEINS

The light harvesting Bchl-protein complexes responsible for greater than 95% of the interaction of Bchl with light are of singular significance (15). These protein species found in Rps. sphaeroides and other members of the Rhodospirillaceae are small, integral membrane proteins (4,13). Their synthesis is controlled by both oxygen and light intensity. Therefore, an understanding of the regulation and synthesis of these proteins, their structure, function and interaction with Bchl is of immeasurable importance if we are to understand the biosynthesis, development and mode of action of photosynthetic tissue. As such we have set about to identify and isolate the genetic determinants involved in both the regulation and primary structure of these very important cellular proteins.

In order to proceed to clone these genetic determinants it was necessary for us to develop two independent lines of inquiry. First, we had to illucidate methodology suitable for the detection of these polypeptides in Escherichia coli. Since we have no selection for such determinants we had to develop a brute force screening procedure. Secondly, we had to purify these polypeptides to homogeneity in order that they can be unambiguously identified and to serve as the basis of our screening procedure.

TABLE I

Molecular Weight Determinations of Polypeptides 15A, 15B, and 15C

Polypeptide	Molecular Weights		
	Sedimentation Equilibrium	Electrophoretic Mobility	Amino Acid Composition
15A	12,000 ± 10%	13,600 ± 5%	11,900
15B	8,800 ± 9%	9,500 ± 5%	9,400
15C	8,900 ± 11%	9,500 ± 5%	9,100

Purification of LH-Proteins

Despite the very hydrophobic nature of these proteins, Dr. Larry Cohen purified the major LH-proteins from Rps sphaeroides without the use of detergents (1). In order to accomplish these ends Dr. Cohen first identified the major polypeptides in isolated B800-850, and B875 spectral complexes derived from Rps sphaeroides, see Figure I. The B-800-850 complex contains two major polypeptides (designated 15B and 15C) with pI's of 5.5 and 6.0 respectively. The major polypeptide from the B875 complex (designated 15A) has a pI of approximately 5.0. An examination of the same protein species on SDS-PAGE, Figure 2, reveals that the B800-850 polypeptides migrate at the dye front whereas the major B875 polypeptide migrates just behind the B800-850 proteins. Note the absence of the B875 polypeptide from fraction 3 of the R-26 mutant strain but the presence of the B800-850 proteins in fraction 4 of R-26. Partial purification of these proteins from wild type 2.4.1 is also shown. These three polypeptides were ultimately purified without the use of detergents and it was shown that in both SDS-PAGE systems and on isoelectric focusing gels that the purified proteins were identical to those found in the native complexes, see Figure 3.

Molecular weights of approximately 9,000 for the two proteins from the B800-B850 complex (15B and 15C) are obtained when determined by three independent methods (2) and Table I, whereas the B875 protein (15A) has a molecular weight of nearly 12,000. Further, both B800-850 proteins have blocked amino terminae and carboxyl terminal glycine residues, whereas the B875 protein has an amino aspartic and a carboxyl serine, Table II. The B800-850 proteins differ in only four amino acid residues out of approximately 100, namely: leucine, proline, arginine and methionine, Table III, and by peptide mapping we can conclude that they show better than 75% sequence homology but they are clearly different, Figure 4.

TABLE II

End Group Determinations on Polypeptides 15A, 15B, and 15C

POLYPEPTIDE	N-TERMINI	C-TERMINI
15A	ASP	SER
15B	BLOCKED	GLY
15C	BLOCKED	GLY

TABLE III

Amino Acid Compositions of Polypeptides 15A, 15B, and 15C

AMINO ACID	MOLE %	MOLE NO.	MOLE %	MOLE NO.	MOLE %	MOLE NO.
ASP	11.2	12.9	6.5	5.9	6.3	5.7
THR	7.3	8.4	7.3	6.6	7.3	6.6
SER	5.6	6.4	6.1	5.5	6.0	5.4
GLU	12.0	13.9	9.4	8.5	9.5	8.6
PRO	6.8	7.9	5.3	4.8	4.5	4.0
GLY	8.8	10.2	12.0	10.8	12.4	11.3
ALA	9.5	10.9	14.4	13.0	14.3	12.9
VAL	1.5	1.7	8.3	7.5	8.2	7.5
MET	7.0	8.1	2.3	2.1	3.0	2.7
ILE	2.2	2.5	4.1	3.7	4.1	3.7
LEU	10.4	12.0	10.8	9.8	11.6	10.5
TYR	1.0	1.2	0	0	0	0
PHE	5.4	6.2	4.1	3.7	3.9	3.6
HIS	0.4	0.4	3.1	2.8	3.1	2.8
LYS	9.8	11.3	4.2	3.8	4.2	3.8
ARG	1.2	1.3	2.3	2.1	1.3	1.1
TRP	--	3	--	2	--	2
CYS	0	0	0	0	0	0

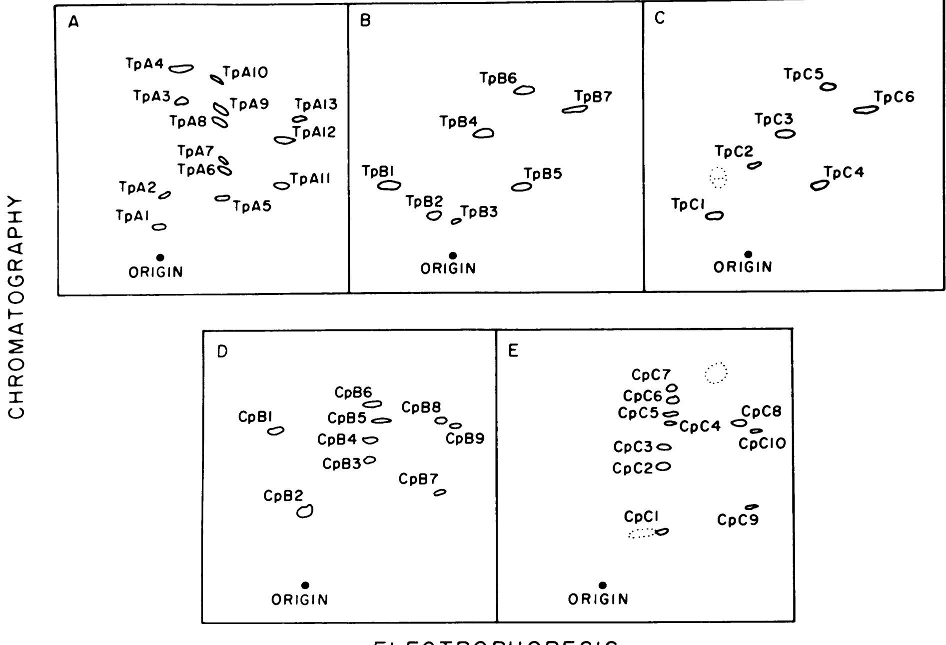

Figure 4. Two dimensional peptide maps of non-detergent purified light harvesting polypeptides. A is B875 tryptic map; B and C B800-850 tryptic maps; D and E are B800-850 chymotryptic maps.

Cloning Bacteriochlorophyll-Binding Proteins

The next thing that Dr. Yen and Mr. Fornari proceeded to do was to make several clone banks employing pBR322 as vector and cloning into either the *amp* or *tet* genes. These procedures need not be described further.

In order to screen for clones of *Escherichia coli* containing the structural genes for those polypeptides Dr. Yen developed a radioimmunological screening procedure whereby iodinated antibody had to diffuse to the suspected antigen, which was fixed on a solid matrix, see Figure 5. The rationale employed was that because of the very hydrophobic nature of these polypeptides (between 60-70% apolar amino-acids) they would not be freely diffusable. Therefore clones were grown and lysed on Whatman number 42 filter paper which is a hydrophobic matrix permitting hydrophobic proteins to remain in place as described by Raetz. In order to prevent non-specific IgG binding to the filter paper containing lysed clones, the paper was soaked in a solution of BSA and dried before addition of IgG. Positive clones appeared as darkened regions conforming in location to a particular colony against a very light gray background. Only

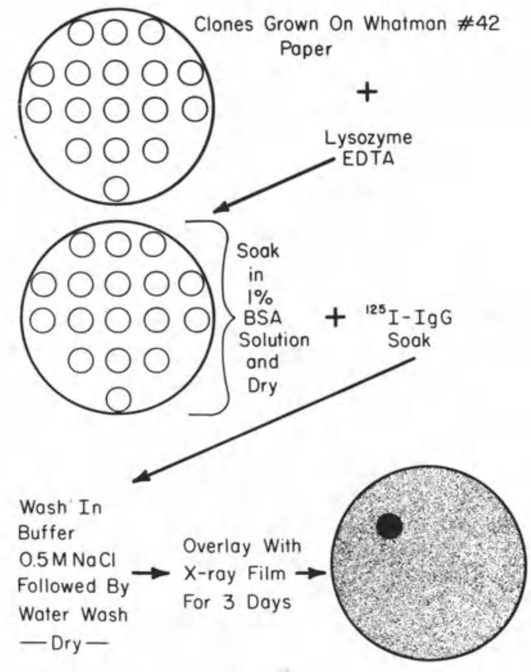

Figure 5. Immunoscreening procedure.

lysed colonies showing a positive reaction following three successive screening procedures were chosen for further study. Figure 6 shows the results of such an experiment with appropriate controls.

The next series of steps involves the characterization of those clones which gave a positive signal with either a multivalent antibody or antibody directed against purified proteins. In Figure 7 we show an agarose gel profile of the chimeric DNA's isolated from the various clones. Lane D is a simple mixture of known plasmid DNA's serving as a series of molecular weight standards. The cloned fragments range in size from 0.25×10^6 - 3.6×10^6 Daltons. These values were independently verified by comparing the size of the reisolated cloned fragments to a Hind3-EcoR1 digest of phage lambda DNA. In Figure 8 are shown minicell lysates derived from cultures grown under low aeration and containing several of the chimeric plasmids previously discussed. Lanes A and F represent protein standards. Lane L is a minicell lysate derived from a pBR322 containing strain.

In Figure 9 are shown several high aeration minicell lysates as well as several maxicell lysates containing some of the plasmids described for Figure 8. Lanes A and L are pBR322 in minicells.

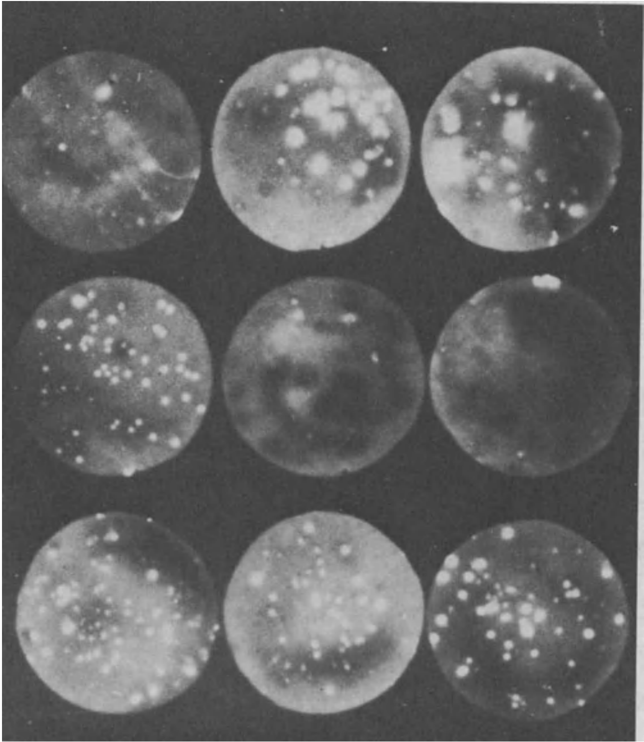

Figure 6. Radioautogram of filter paper ^{125}I-immunoscreening procedure. Proceeding from the upper left to right, the first four filter discs contains a mixture of Rps sphaeroides and E. coli. The last two discs of the middle row are radioautograms of E. coli containing cloned Rps sphaeroides DNA. There are several hundred colonies on a disc. Note that clones which are positive in the screening procedure appear as bright spots. The bottom row is a mixture of a clone giving a positive reaction as described above with a large excess of E. coli cells containing only plasmid pBR322. Because the image has been reversed the positive signals appear as bright spots.

Lane F is pBR322 in maxicells and Lane E is pRK290 in maxicells, a 13.2 x 10^6 dalton plasmid, used here as a standard.

Plasmid M78A, containing an 840 bp insert into the Pst site of the amp gene of pBR322 yields divergent results when the minicell products are derived from low vs. high aeration culture. In Figure 8 (low aeration) the size of the gene product is approximately 28,000 daltons. In Figure 9 (high aeration) the size of the gene product is approximately 52,000-54,000 daltons. Further, the

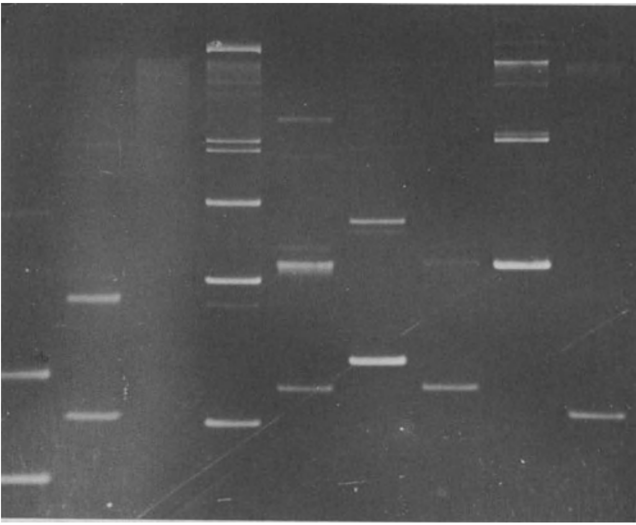

Figure 7. Agarose gel of recombinant plasmids containing pBR322 and Rps. sphaeroides chromosomal DNA. Lane A, plasmid M57C; lane B M61H (2.85×10^6 daltons); lane D, molecular weight standards: from top to bottom, pRK290 (13.2×10^6 daltons), pMW79 (8.4×10^6 daltons), RSF1010 (5.5×10^6 daltons), pBR322 (2.7×10^6 daltons); lane E, M56C, (3.2×10^6 daltons); lane F, I4, (3.6×10^6 daltons); lane G, M78A, (3.2×10^6 daltons), lane H, HindIII, ($6,2 \times 10^6$ daltons); lane I pst 13, (2.85×10^6 daltons).

difference between low and high aeration minicells can be shown by comparing lanes J of Figure 8 and K of Figure 9. The former is a low aeration minicell lysate containing plasmid M61H. Notice the very high background resulting from poor minicell production. Lane K of Figure 9 although a maxicell preparation is identical to a high aeration minicell preparation containing plasmid M61H. Note the low background and the presence of the gene product at 37,000 daltons.

Clone I4, containing a 1×10^6 Dalton insert into the Sal I site of the tet gene of pBR322 gives two new protein products, at 25,000 and 17,000 daltons. We are presently attempting to relate these specific gene products derived from Rps. sphaeroides DNA to known proteins of the PM. The methods which must be employed are protein chemistry and immunochemistry in order that we may obtain an unambiguous identification of these gene products.

However, we would like to return your attention to clone M78A showing gene products of 52,000-54,000 Daltons in high aeration

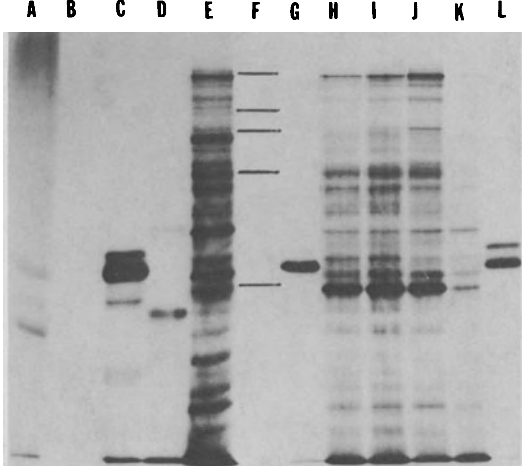

Figure 8. Autoradiograph of SDS-PAGE gel showing ^{35}S-labeled gene products from plasmid DNA in minicells of E. coli cultured under conditions of low aeration. Lane A, ^{35}S-labeled reaction center polypeptides from Rps. sphaeroides, 28,000, 24,000, 21,000. Lane B, control lysate from minicells without plasmid DNA; lane C, lysate from minicells with I4 plasmid; lane D, pst 13 plasmid gene products, lane E, plasmid Hind III gene products; lane F, molecular weight standards: phosphorylase b, 94,000 daltons, bovine serum albumin, 68,000 daltons, catalase, 57,500 daltons, ovalbumin, 43,000 daltons, and α-chymotrypsinogen, 25,700 daltons; lane G, plasmid M78A labeled gene products; lanes H, I, J, and K are labeled gene products from plasmids M57C, 405D, M61H, and M56C respectively; lane L, pBR322 gene products.

minicells and 28,000 daltons in low aeration minicells. Based upon the size of the cloned DNA (840 bp) there is sufficient information to code for a protein of approximately 30,000-32,000 daltons. Therefore we must consider the source of additional protein in the high aeration minicells. The fragment of the β-lactamase which could be produced is approximately 20,000 Daltons. Therefore, we believe that we are seeing a fused gene product in high aeration minicells (52,000 daltons) and a fragment of this product is low aeration minicells (28,000 Daltons). In Figure 10 are the gene products from an in vitro protein synthesizing system. We call your attention to lane 4 which is plasmid M78A. Notice the two major products whose size are approximately 52,000 and 54,000 daltons. The larger is the predominant species. We suggest that this is the fused gene product described earlier but in additon containing the β-lactamase signal sequence. We believe that the 52,000 dalton species is identical to the major product observed in high aeration minicells.

A B C D E F G H I J K L

Figure 9. Autoradiograph of an SDS-PAGE gel containing ^{35}S-labeled gene products from high aeration minicells and maxicell lysates. Lane A and L, pBR322 in minicells; lane B, plasmid HindIII in minicells; lane C, M78A in minicells; lane E, pRK290 from a maxicell lysate; lane F, pBR322 from a maxicell lysate; lane J, M57C from maxicells; lane K, M61H from maxicells.

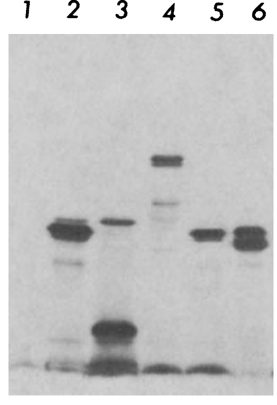

Figure 10. SDS-polyacrylamide gel patterns of proteins synthesized in an *E. coli* cell-free S-30 system. DNA templates are: lane 1, no DNA control; lane 2, pBR322; lane 3, pSL25; lane 4, clone M78A; lane 5, clone Hind III. Lane 6 is the corresponding gel pattern of proteins synthesized in *E. coli* minicells containing pBR322 plasmid.

There is mounting evidence that Rps. sphaeroides DNA is either not expressed or only poorly expressed in E. coli unless, as we believe from the results described here, the Rps. sphaeroides DNA can be fused in phase and transcribed from an E. coli promoter. We have additional evidence that the gene products which we have observed for M78A as well as other clones described here all represent fused gene products. Because the genes to which the Rps. sphaeroides DNA have been fused themselves give rise to membrane associated products, the processing of these proteins or the lack of such processing can lead to multiple forms of the product proteins. The conditions of minicell growth are critical to which form of the gene product will predominate.

In conclusion, evidence indicates that we have cloned important gene products from Rps. sphaeroides. However, their ultimate identification awaits solution to the problems discussed above.

ACKNOWLEDGEMENTS

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DISCUSSION

Q. REEVE: When you say you see 4 mini-cells under the microscope, what do you mean?

A. KAPLAN: No, we monitor the yield of Minicells microscopically.

ACINETOBACTER: A TALE OF TWO GENERA

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INTRODUCTION

The name Acinetobacter was first applied in 1954 by Brisou and Prévot (5) to nonmotile strains of Achromobacter, the latter group of bacteria representing an earlier classification of gram-negative saprophytes that could be distinguished from other similar bacteria because of their lack of pigmentation (16). In subsequent studies of acinetobacters it was shown that some of them were oxidase-positive and others were oxidase-negative (7). As a result of several investigations of the oxidase-negative acinetobacters an official designation was made of these organisms as species of Acinetobacter calcoaceticus (26). The oxidase-positive acinetobacters were then shown to be unrelated to the oxidase-negative acinetobacters (22) and can no longer be considered strains of the genus Acinetobacter. Both groups of acinetobacters contain members that can be manipulated genetically and each will be considered in this discussion.

PHYSIOLOGY

Although a review of the oxidase-negative acinetobacters has appeared recently (20) some of the physiological characteristics of members of this group will now be discussed. Strains of Acinetobacter calcoaceticus are all aerobic, gram-negative, nonmotile, nonpigmented coccobacilli. They are oxidase-negative, catalase-positive and, because most of them fail to reduce nitrate, they have been referred to as strains of Bacterium anitratum.

Strains of A. calcoaceticus are ubiquitous and can be isolated readily from soil (3,36), water (3,36) and from various locations in

humans (12) and animals (8). I first encountered acinetobacters when enriching for bacteria capable of utilizing acetoin or 2,3-butanediol as a sole source of carbon and energy (18). At approximately the same time a group of French workers reported the isolation of bacteria, which they called strains of Neisseria winogradskyi, using 2,3-butanediol-nitrate enrichment cultures from 18 out of 25 soil samples examined (25). These organisms were subsequently shown to be strains of A. calcoaceticus (4,19). In a study of the enrichment of acinetobacters from soil and water, Baumann was able to isolate these bacteria from 28 of 30 soil samples and from 29 of 30 water samples tested (3). He demonstrated that isolation of acinetobacters by enrichment was favored by vigorous aeration and slightly acid pH (3). Studies in our laboratory have shown that optimum growth in a malate-mineral medium takes place at a slightly acid pH (Figure 1). Semiquantitative studies by Baumann (3) enable him to estimate that at least 0.001% of the bacterial strains in soil and water capable of growth on a complex medium were acinetobacters.

In addition to their occurrence in soil and water acinetobacters are also found frequently on the mucous membranes and the skin of humans and other animals (2,8,12). Acinetobacters have been shown to cause severe infection in debilitated individuals and are common causative agents of nosocomial infections (11,12). Because the oxidase-negative acinetobacters are encountered frequently but have no unique distinguishing characteristics, it is not surprising that various workers have placed strains of this group into 15 different genera (4). Names usually employed included Acinetobacter, Achromobacter, Bacterium, Moraxella, Neisseria, Micrococcus, Diplococcus, Herellea, Mima, Alcaligenes, Cytophaga as well as others.

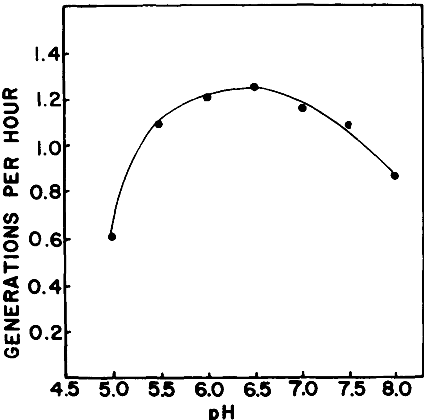

Figure 1. Optimum growth pH for Acinetobacter calcoaceticus, strain BD413. Growth rate as a function of initial pH in a malate-mineral medium.

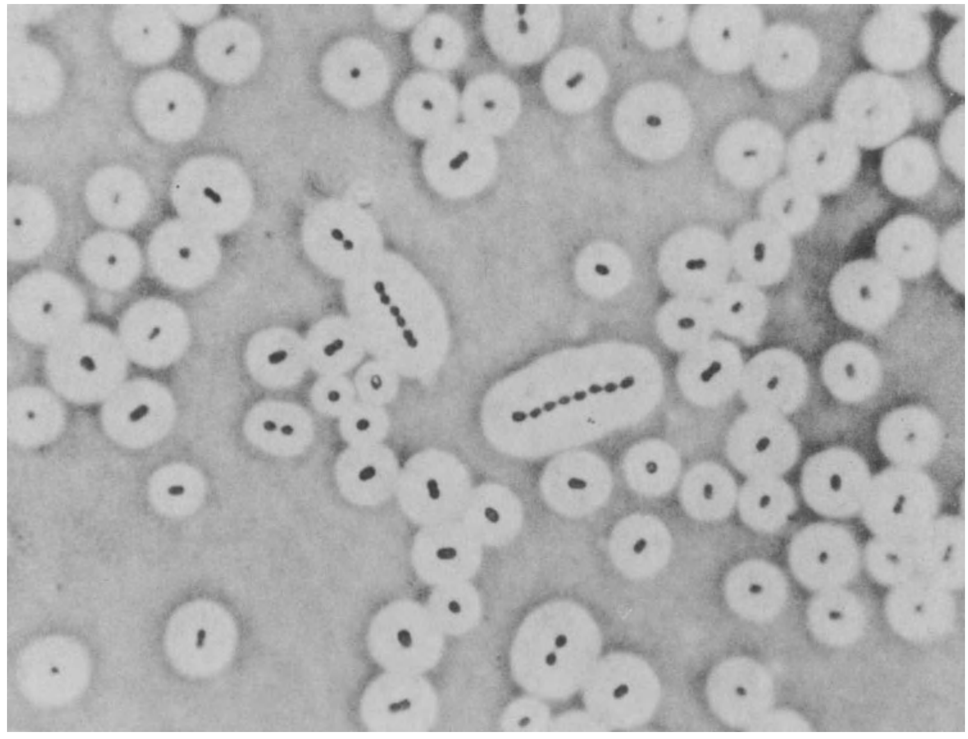

Figure 2. Phase contrast microscope photograph of Acinetobacter calcoaceticus, strain BD4. A wet mount in India ink of cells grown in a glucose-mineral medium for 24 hours.

Figure 2 is a phase contrast microscope photograph of a wet mount of a highly encapsulated strain of A. calcoaceticus suspended in India ink to reveal the capsule. Many strains of A. calcoaceticus are highly encapsulated but most of them possess what appears to be a microcapsule (Figure 3) which is most probably lipopolysaccharide in composition. During exponential growth cells tend to be plump rods (4). These change to cocci when growth enters the stationary phase, cells occurring commonly in pairs but also as single cells as well as chains of cells. Figure 4 is an electron microscope photograph of a thin section of A. calcoaceticus illustrating the three morphologically distinguishable layers characteristic of the cell envelopes of gram-negative bacteria.

Figures 5 and 6 show that the optimum temperature of a typical strain of A. calcoaceticus is between 32-35°C. Minimum generation times less than 40 minutes have been obtained for growth in a malate-mineral medium.

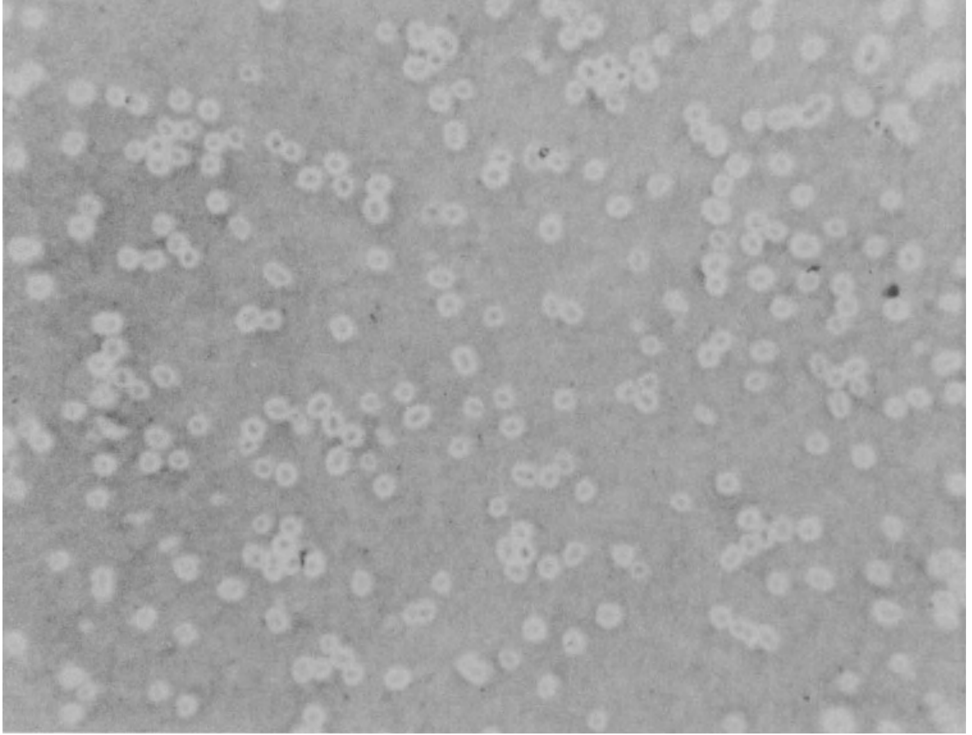

Figure 3. Phase contrast microscope photograph of *Acinetobacter calcoaceticus*, strain BD413. A wet mount in India ink of cells grown in a glucose-mineral medium for 24 hours. Strain BD413 is an unencapsulated mutant of strain BD4.

The large majority of strains of *A. calcoaceticus* isolated from soil, water, sewage, and from human infections are capable of growing aerobically in a mineral medium containing a single carbon source, such as acetate, with ammonia or nitrate salts as the source of nitrogen (4). It has been shown that most strains are able to utilize a wide variety of organic compounds as carbon and nitrogen sources (4). Although acinetobacters generally oxidize their organic substrates to completion it has been demonstrated that certain metabolic intermediates occasionally accumulate in growth media. For example, cetyl palmitate was shown to accumulate during growth in a n-hexadecane-mineral medium (30). Growth in an acetoin-mineral medium resulted in accumulation of acetic acid and acetylbutanediol, both intermediates in the bacterial dissimilation of 2,3-butanediol and acetoin (21). In each of these examples accumulation of intermediates occurred when the medium pH became acid and such accumulations may be the result of inability to oxidize these compounds further under these conditions.

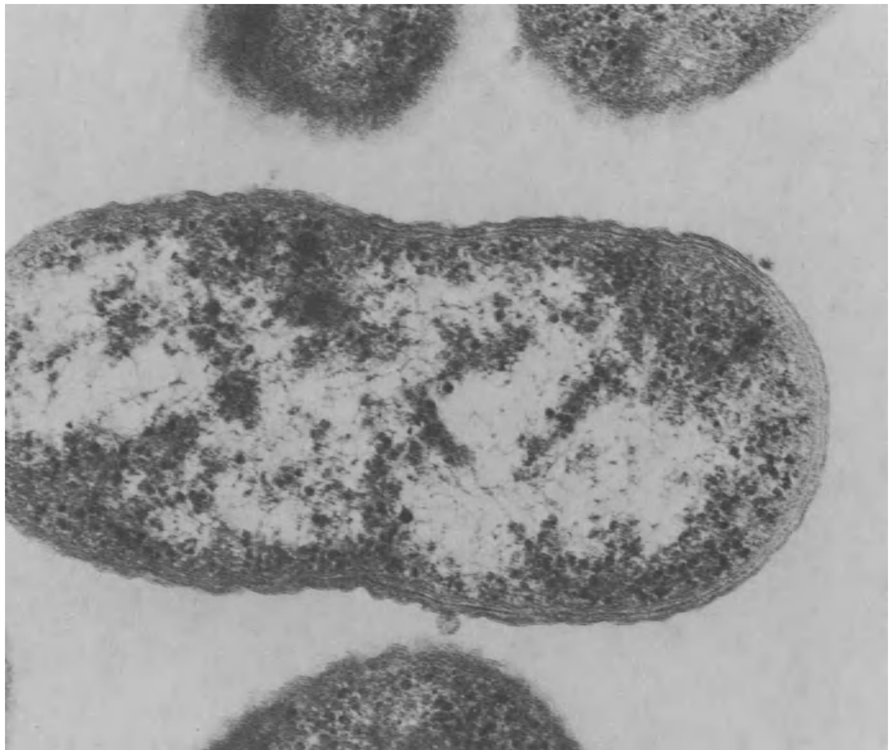

Figure 4. Electron microscope photograph of a thin section of *Acinetobacter calcoaceticus*, strain BD413. Photograph taken by D.C. Swartzendruber.

GENETICS

Transformation

During studies of capsule biosynthesis by an encapsulated *Acinetobacter* strain (strain BD4) it was observed that growth of two stable nonidentical unencapsulated mutants together in the same medium gave rise to the appearance of small numbers of fully encapsulated cells (23). This result was shown to be due to transformation of one mutant by DNA from the other mutant, DNA appearing in the medium because of a small amount of cell lysis (23). In this way it was discovered that the *Acinetobacter* strain under study was highly competent for genetic transformation. A study of several hundred independently isolated *Acinetobacter* strains revealed that two of them were weakly competent but that none of them were as highly competent as the strain in which transformation was originally discovered (strain BD4) (20). Studies in other laboratories (1,9) have shown that two other strains are also weakly competent.

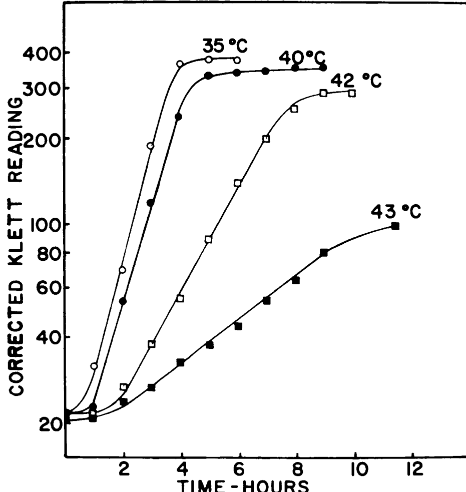

Figure 5. Culture turbidity as a function of time of growth of Acinetobacter calcoaceticus, strain BD413 in a malate-mineral medium at various growth temperatures. The Klett readings of dense cell suspensions were corrected for departure from linearity.

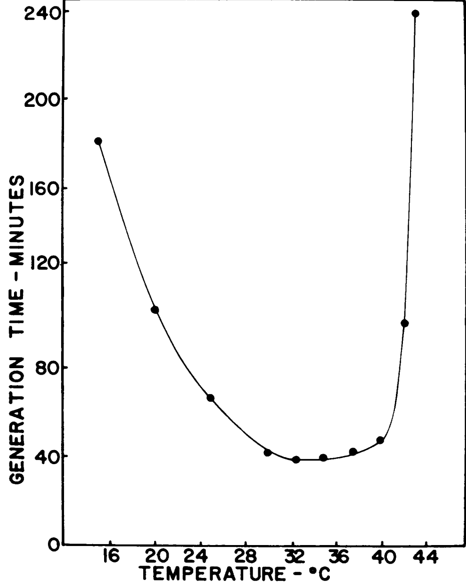

Figure 6. Generation time of Acinetobacter calcoaceticus, strain BD413 as a function of growth temperature. The generation times were taken from the growth curves of Figure 5, as well as from growth curves not shown.

Transformation in the highly competent *Acinetobacter* strain occurs readily in any growth medium and can be performed on semi-solid media or in liquid culture. It has been demonstrated that cells are competent over the entire growth period but there is a peak of competence in the early part of the exponential growth phase (1,9). Using auxotrophs of the highly competent strain it has been shown that some of these mutants can be transformed to prototrophy with DNA from all strains of *Acinetobacter* examined (several hundred) (19). This finding is the basis of a transformation assay for identification of strains of *A. calcoaceticus* (19) and this procedure has been applied to identification of acinetobacters isolated from clinical materials (6). DNA samples from other unrelated bacteria have failed consistently to transform *Acinetobacter* auxotrophs to prototrophy (19). The report that DNA from *Pseudomonas aeruginosa* can transform a citrate synthase deficient mutant to produce functional enzymes (37) is most likely the result of rare spontaneous reversions since citrate synthases in the presumed transformed cultures do not have properties characteristic of *P. aeruginosa* citrate synthase. It has been reported that the presence of cyclic AMP increases the frequency of transformation of competent *Acinetobacter* (1).

Transduction

Since acinetobacters are isolated readily from sewage (36) it is not surprising that a large variety of *Acinetobacter* phages can be isolated from sewage (13,35). Although most *Acinetobacter* phages are lytic, one temperate phage has been isolated (13). This phage readily lysogenizes the host strain and is able to mediate generalized transduction (13). *Acinetobacter* phages tend to be host strain specific. Since strains of *Acinetobacter* have been shown to possess a large number of different surface antigens (27) this fact may account for the narrow host specificity of *Acinetobacter* phages (13).

Conjugation

In 1976 Towner and Vivian (32,33) showed that the resistance plasmid RP4, upon transfer from *Escherichia coli* K12 to *Acinetobacter*, could mobilize the *Acinetobacter* chromosome and transfer chromosomal genes from the strain harboring the plasmid to other strains of *Acinetobacter*. Linkage of chromosomal markers was demonstrated as well as polarity of marker transfer (31). With plasmid-carrying strains which donate the chromosome in different directions it was shown that the linkage map in *Acinetobacter* is circular (31,33). Using RP4 mediated conjugation Towner (31) mapped 23 different mutations in a circular linkage group in one strain of *Acinetobacter*. Other plasmids of the P incompatibility group can mobilize the *Acinetobacter* chromosome (34). Plasmids of the C,F,I, and W incompatability groups from *E. coli* and the P2 incompatibility group from

P. aeruginosa could not be transferred to Acinetobacter and could not, therefore, be tested for possible mobilization of the Acinetobacter chromosome (34). Acinetobacters isolated from patients in hospitals appear to harbor plasmids and one of these, designated pAV1 has been shown to mobilize chromosomal markers and is transmissible to other strains of Acinetobacter (14,15).

Olsen and Shipley (28) demonstrated that plasmid R1822, originally obtained from P. aeruginosa, can be transferred to Acinetobacter where it can be maintained and also be donated to other bacteria. Ditta et al., (10) recently described a DNA cloning vehicle for gram-negative bacteria that has been constructed from the antibiotic resistance plasmid RK2. This plasmid is capable of being transferred to strains of Acinetobacter where it can be maintained.

OXIDASE-POSITIVE ACINETOBACTERS

The oxidase-positive acinetobacters are largely psychrotrophic with an optimum growth temperature of 20°C. They occur naturally as commensals on the skins of fish and are frequently encountered in processed foods. Because they are unusually radiation resistant these bacteria appear following gamma irradiation of fish (24), meats (17), and other foods. A study of the oxidase-positive acinetobacters has revealed that many strains are competent for genetic transformation (22). The existence of a transformation assay for identification of psychrotrophic acinetobacters has made it possible to recognize strains belonging to this group as well as to demonstrate that the oxidase-positive acinetobacters are unrelated to the oxidase-negative species and are also unrelated to the Moraxellae, a large group of gram-negative oxidase-positive coccobacilli found to occur frequently on mucous membranes of humans and other animals (12). Because of their lack of relatedness to other similarly appearing organisms we will propose shortly that the oxidase-positive acinetobacters be classified as strains of Psychrobacter immobilis.

CONCLUDING REMARKS

According to the definition of Brisou and Prévot (5) the genus Acinetobacter includes both oxidase-negative and oxidase-positive strains. Based upon the results of studies of classical toxonomic properties and genetic relationships it has now been agreed that the genus Acinetobacter include only the oxidase-negative strains (26).

Interest in Acinetobacter has been increasing in recent years and many reports concerning the physiology, metabolism and genetics of these organisms have appeared (20). Because of their rapid

growth and ready utilization of a great many substrates the acinetobacters are proving to be suitable organisms for study of basic microbiological problems. The ability of acinetobacters to degrade many organic compounds may make it possible to isolate metabolic intermediates of commercial interest through the use of suitable mutants or by other techniques. It is of particular interest that, unlike the situation for the enteric bacteria, genes for enzymes of several biosynthetic pathways do not occur together in operons in *Acinetobacter* but are distributed in various regions of the chromosome (29,31). Future studies of the regulation of these enzymes may reveal new mechanisms for such control.

The capability of using the processes of transformation, transduction and conjugation with various strains of *Acinetobacter* makes possible a large variety of genetic manipulation with these bacteria. Since a suitable cloning vehicle is now available for *Acinetobacter* (10) it should be possible to clone various genes of interest in members of this genus. It could prove most useful to perform cloning studies with the highly competent *Acinetobacter* to make possible further genetic modifications of such strains.

Although very few physiological or genetic studies have as yet been carried out with the psychrotrophic oxidase-positive acinetobacters, these organisms are most promising subjects for future investigations. If cloning vehicles can be found for these bacteria there would be little danger of engineered strains taking up residence in humans or higher animals since these strains do not grow at body temperature. They do, however, grow extremely well at room temperature in very simple media (22).

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DISCUSSION

Q. CHAPMAN: Are you aware of any naturally-occurring degradative plasmids in *Acinetobacter*?

A. JUNI: I am not aware of any.

GENETIC ALTERATION OF *ZYMOMONAS MOBILIS* FOR ETHANOL PRODUCTION

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INTRODUCTION

Recent interest in ethanol as a potential fuel or fuel supplement has stimulated research into various aspects of the fermentation process. Techniques such as continuous fermentation, vacuum distillation and methods for cell recycle have been investigated (6,10,11), but another important area being studied is that of strain selection and improvement for maximum productivity.

Traditionally, yeasts have been used for industrial ethanol fermentations. However, in a recent review of the biology of *Zymomonas*, Swings and DeLey (31) suggested that since these organisms ferment rapidly and efficiently they might be promising agents for large-scale production of ethanol.

Zymomonas mobilis is a Gram-negative bacterium which is used in the tropics to make palm wines and which also causes cider and perry sickness (2,20,23,25,31). Unlike *Saccharomyces*, which metabolizes hexoses to ethanol via glycolysis, *Zymomonas* uses a modification of the Entner-Doudoroff pathway to rapidly produce up to 1.9 moles of ethanol per mole of glucose fermented (12,13,31). Furthermore, *Zymomonas* can grow in high sugar and ethanol concentrations; Swings and DeLey (31) found that half their strains grew in media with 40% w/v glucose or 10% v/v ethanol.

Most previous studies of *Z. mobilis* have been concerned with the isolation, physiology and taxonomy of the organism. However several authors have tested the growth and ethanol yields on low concentrations of glucose. Belaich and Senez (3) obtained ethanol

yields of between 0.39 and 0.40 g/g glucose in complex, minimal and synthetic media with 2g/l glucose. Dawes et al. (8) obtained a yield of 0.43 g/g with 20g/l glucose. Using another strain of Z. mobilis in media with 46g/l glucose, Millis (22) reported an ethanol yield of 0.49g/g. These values represent a yield of between 76% and 96% of the theoretical yield of 0.51g/g, and gave some indication of the high productivities achievable with this organism.

Recently, data have been reported on the fermentation of high concentration sugar media which are relevant for assessing the economic aspects of the Zymomonas fermentation as an industrial process. For example, Lavers et al. (15) have compared Z. mobilis strain ATCC 29191 (ZM6) and Saccharomyces cerevisiae in batch and continuous cultures. They found that although yeast and Z. mobilis grew at comparable rates in 10% glucose medium, the specific rates of glucose utilization and ethanol production were several times higher for Zymomonas (15). The ethanol yield with Zymomonas was approximately 98% of the theoretical value.

Similar conclusions have been reached in this laboratory, where a different strain of Z. mobilis (strain ZM1) has been compared with yeast (16,26). Saccharomyces uvarum strain ATCC 26602, selected for its flocculant properties and ability to tolerate high sugar and ethanol concentrations (28) was compared with Z. mobilis strain ZM1

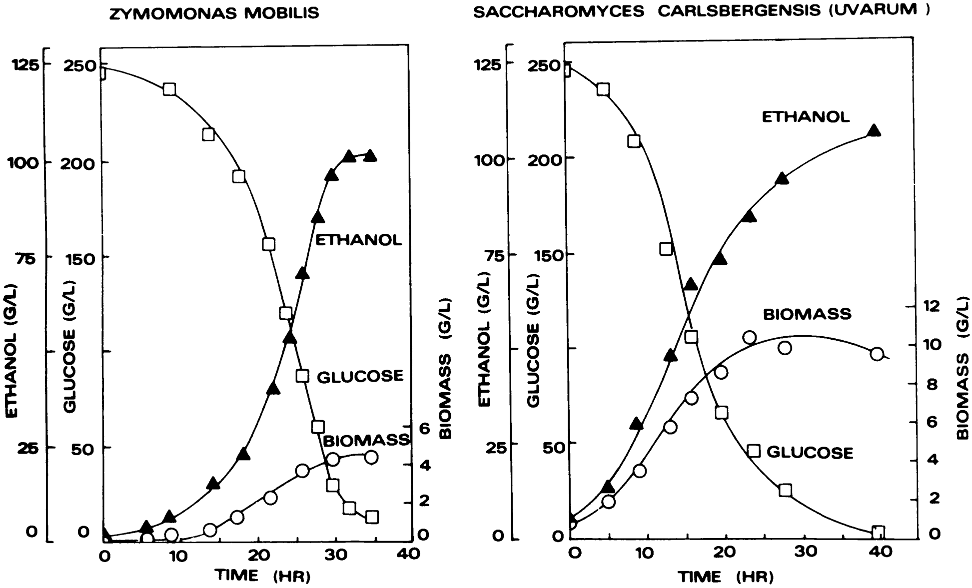

Figure 1. Comparative kinetics of Z. mobilis strain ZM1 and S. uvarum strain ATCC 26602 in 250g/l glucose medium.

(ATCC 10988) in batch culture with between 100g/l and 250g/l glucose media (Figure 1) (26). From these studies, it was apparent that Z. mobilis had several advantages over yeast for ethanol production. These included significantly higher rates of glucose uptake and ethanol production (double the glucose rate and three times the ethanol rate in 250g/l glucose medium), higher ethanol yields (93 to 97% of the theoretical yield compared with 86% for yeast), and comparable or higher ethanol tolerance (26).

In continuous culture without cell recycle, concentrations of up to 65g/l ethanol were obtained, although steady states were only achieved at ethanol levels of up to 60g/l; higher ethanol levels caused oscillatory behavior (16).

When strain ZM1 was tested in a continuous culture system with cell recycle by a Millipore tangential flow microfiltration system, ethanol productivities of up to 120g/l/h were obtained (27). This is appreciably higher than reported values for yeast, where productivities of 36g/l/h without vacuum and 82g/l/h with vacuum are the maximum values reported (6,27).

In view of the encouraging results obtained in this laboratory with strain ZM1, attempts were made to procure even better strains of Zymomonas for ethanol production, both by screening a variety of natural isolates and by the development of methods for genetic manipulation of these strains.

STRAIN SELECTION

In a review of the biology of Zymomonas by Swings and DeLey (31), forty different isolates from various parts of the world were compared for a number of properties, including ethanol tolerance, temperature tolerance, growth in high sugar concentrations, use of different sugars and flocculation. Considerable variation between the strains was observed, as can be seen from the summary in Table I. Because of this wide variation, it seemed likely that there would be better strains of Zymomonas available than those originally chosen for kinetic studies. Therefore, strains isolated from a number of different locations were compared for their suitability for fermentation.

Viikari et al. (32) compared strain ZM1 (ATCC 10988) with seven brewery isolates, and found that several of these isolates produced more ethanol and were more temperature tolerant than strain ZM1.

In this laboratory, eleven strains from different locations were compared. The strains tested are listed in Table II, together with their sources.

TABLE I
Properties of Forty Different *Zymomonas* Strains

Property	Percent of Strains with Property
Growth on glucose	
20% w/v	100
40% w/v	54
Growth on Sucrose	48
Growth on Starch	0
Growth at temp. (°C)	
30	100
34	97
36	97
38	74
40	5
Growth in presence of ethanol (%v/v)	
5.5	100
7.7	73
10.0	47
Flocculant deposit	33

TABLE II
Strains of Zymomonas Used

	Strain	Source
Z. mobilis	ZM1 (ATCC 10988)	Fermenting *Agave* juice
	Ag11	Fermenting *Agave* juice
	3TH Delft	Fermenting *Arenga* sap
	ZM6 (ATCC 29191)	Fermenting *Elaeis* sap
	ZM4 (CP4)	Fermenting sugarcane juice
	B70	Infected British ale
	ZAbi	Beer
	Za10	Beer
Z. mobilis subsp. *pomaceae*	ATCC 29192	Sick cider
	238	Cider
	S30.A	Apple pulp
	S30.2	Cider

In initial studies in test tubes, it was immediately apparent that some strains were unable to use sucrose and would therefore be unsuitable for industrial fermentation of sucrose-based substrates such as molasses or sugar beet. Of the remaining strains, several were observed to have shorter lag periods and to grow faster than the other strains (30). The best four strains, including ZM1, were chosen for more detailed studies.

When the four strains were compared in liquid medium with 200g/1 glucose, strain ZM4 was found to produce ethanol at a considerably faster rate than the other three strains (Table III).

TABLE III
Ethanol Production by *Z. mobilis* Strains Growing on 200g/1 Glucose or Sucrose in Test Tubes

Strain	Ethanol production from: 200g/1 glucose		200g/1 sucrose	
	Rate (G/L/H)	Final conc(g/1)	Rate (g/1)	Final conc(g/1)
ZM1	1.43	60	1.22	56
ZM4	2.00	81	1.21	56
ZM6	1.25	77	0.80	51
Ag11	1.40	55	1.00	39

The final ethanol concentration was also higher with this strain (Table III), suggesting that ZM4 may be more ethanol-tolerant than the other strains. This was confirmed in media with added ethanol; only ZM4 could grow with more than 60g/1 ethanol (30).

In media with 200g/1 sucrose, strains ZM1 and ZM4 gave similar results, both producing ethanol faster than the other two strains (Table III). In a one litre fermenter, the ethanol yields followed the same general trends as in test tubes, with strain ZM4 producing 117g/1 ethanol from 250g/1 glucose medium and 89g/1 ethanol from 250g/1 sucrose medium (19). The reduced yield on sucrose is mainly due to the production of a fructose polymer, levan, from sucrose (9,24,31).

At temperatures above 30°C, all strains grew and produced ethanol more slowly, but once again strain ZM4 appeared the best (Table IV).

TABLE IV
Final Ethanol Concentrations Produced by Z. mobilis Strains from 200g/l Glucose in Test Tubes at Different Temperatures

Strain	Final ethanol concentration (g/l) 30°C	37°C	42°C
ZM1	60	40	0
ZM4	81	52	30
ZM6	77	0	0
Ag11	55	0	0

Since strain ZM4 appeared to be the most promising strain overall, it was compared in more detail with strain ZM1 using a one litre fermenter with pH and temperature control (17,26). In batch culture, it was evident that strain ZM4 was considerably better than ZM1 (Figure 2).

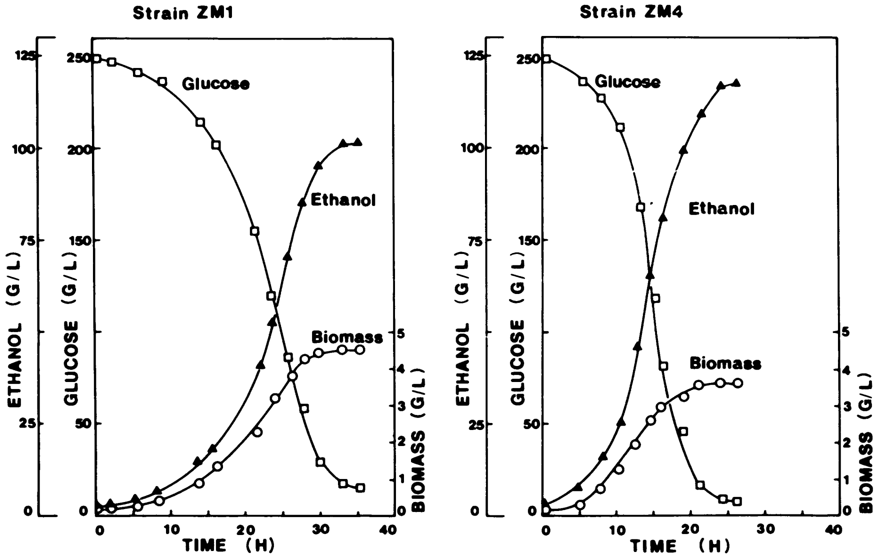

Figure 2. Comparative kinetics for strains ZM1 and ZM4 growing on 250g/l glucose medium.

This difference became more obvious in higher glucose concentrations, with ZM4 being less inhibited by the increased ethanol concentrations (17). In 200g/l glucose medium, ZM4 had specific

glucose uptake and ethanol production rates double those of ZM1, as well as growing considerably faster. The maximum ethanol concentration obtained in batch culture with ZM4 was 127g/l compared with 102g/l for ZM1 (17).

In continuous culture, as in batch culture, strain ZM4 was less inhibited by ethanol than strain ZM1, with higher ethanol concentrations being produced by ZM4 (18). Strain ZM4 has also been tested in an immobilized cell reactor and productivities of up to 53g/l/h have been obtained (14). Arcuri et al. (1) also obtained high productivities with strain ZM1 in an immobilized cell reactor.

STRAIN IMPROVEMENT

Since strain ZM4 was clearly better than ZM1, but there was still room for further improvement of, for example, ethanol tolerance, attempts were made to genetically manipulate ZM4.

Mutagenesis Techniques

Since no attempts to mutate *Zymomonas* had been reported previously, it was first necessary to devise a suitable method of mutagenesis for this organism. Initially, ultraviolet light was tested as a mutagenic agent, selecting for an increased level of colonies resistant to the antibiotic rifampicin. Strains ZM1 and ZM4 normally have a low level of spontaneous mutation to rifampicin resistance of about 10^{-8}, and no increase in this level could be detected after exposure to UV light. However, with nitrosoguanidine (NTG) as the mutagen, a tenfold increase in the number of rifampicin-resistant colonies was obtained. There was considerable strain variation in the level of sensitivity to this mutagen, with some strains only requiring 25mg/l NTG for 60min to give the best levels of mutagenesis, while others needed up to four times this amount. The best rates of mutagenesis were obtained when NTG was added directly to growing cultures in rich medium rather than in buffer.

Selection of an Ethanol-Tolerant Mutant

Having determined the optimum time of exposure to NTG, strain ZM4 was treated with this mutagen and ethanol-tolerant mutants were selected. Strain ZM4 can grow on agar plates with up to 8% v/v ethanol, but after mutagenesis a few cells were able to form colonies on plates with 12% v/v ethanol. These colonies were purified and tested for ethanol production in media with high levels of glucose. The best mutant, ZM48, appeared more ethanol-tolerant in test-tube culture (Table V) but did not show any real improvement in the fermenter.

TABLE V
Final Ethanol Concentrations of Strain ZM4 and Ethanol-Tolerant Mutants Growing on 300g/1 glucose Medium

Strain	Final ethanol conc. (g/1)	Time taken to reach max. ethanol conc. (h)
ZM4	93	108
ZM444	98	110
ZM48	101	101
ZM485	108	110
ZM488	112	132
ZM481	110	84

However, after a second round of mutagenesis on strain ZM48, several colonies appeared on 15% v/v ethanol plates. Some of these produced higher final ethanol concentrations but grew slower than the parent strain (Table V). The best isolate, strain ZM481, showed more promise in test tubes, particularly with high levels of glucose where it produced a higher final ethanol concentration in a shorter time than either ZM4 or ZM48 (Table V). In the fermenter, it was confirmed that ZM481 was a more promising strain than ZM4 at high ethanol concentrations.

In long term continuous culture with cell recyle, ZM4 could sustain 65g/1 ethanol, but it was difficult to maintain a stable recycle operation due to a loss of viability at ethanol levels above 75g/1. The mutant strain ZM481, however, retained a higher viability in high ethanol concentrations (Figures 3 and 4) so that ethanol concentrations could be maintained at 85g/1, a significant increase over the parent strain ZM4.

A comparison of strains ZM4 and ZM481 in the fermenter at different dilution rates clearly shows that ZM481 can maintain a higher ethanol concentration and productivity than ZM4 in continuous culture (Figure 5).

Selection of a Flocculant Mutant

For cell recyle in many of the continuous culture experiments done in this laboratory, a cross-flow membrane system has been used (18,27). However, scale-up of such a system for industrial use may be impractical due to membrane clogging by molasses or starch hydrolysates, and therefore other cell recycling systems have been

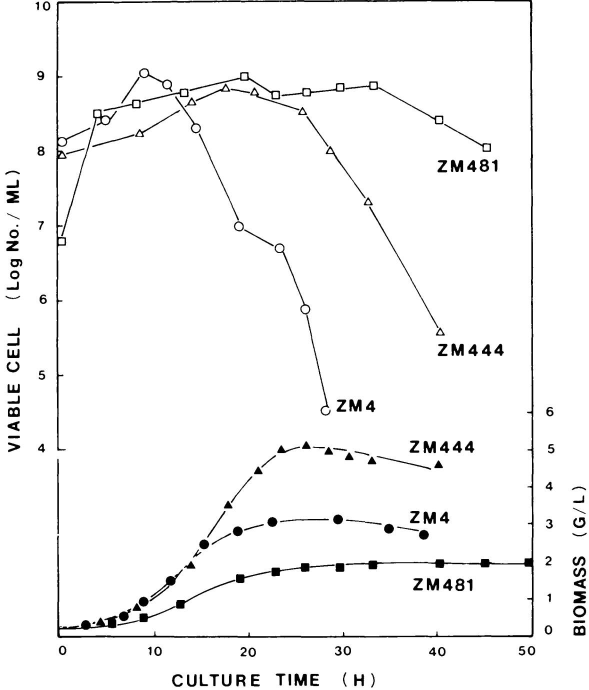

Figure 3. Cell viability with 300g/l glucose in the fermenter.

examined. With yeasts, spontaneously flocculating strains are available which can be recycled on the basis of this property. Although many strains of Zymomonas were reported to be flocculant (31), their flocculation is insufficient for utilization in cell recycling. Strain ZM4 does not flocculate spontaneously, but after NTG mutagenesis a highly flocculant mutant was isolated. This mutant forms small granular flocs in the fermenter which settle out as flocs up to several millimeters in diameter within a minute or so after agitation is stopped. This mutant, ZM401, shows promise in a continuous system with cell recycling by means of an external settling tank, and also in semi-batch culture where productivities of 50g/l/h have been obtained.

Selection For Good Growth on Molasses

Although strain ZM4 and its derivatives grow well on sucrose media, growth and ethanol production from molasses is poor. This is probably due to the high concentrations of Mg^{++} and K^{+} ions in molasses. To partially overcome this problem, strain ZM48 was mutated with NTG and plated on media containing a level of molasses which

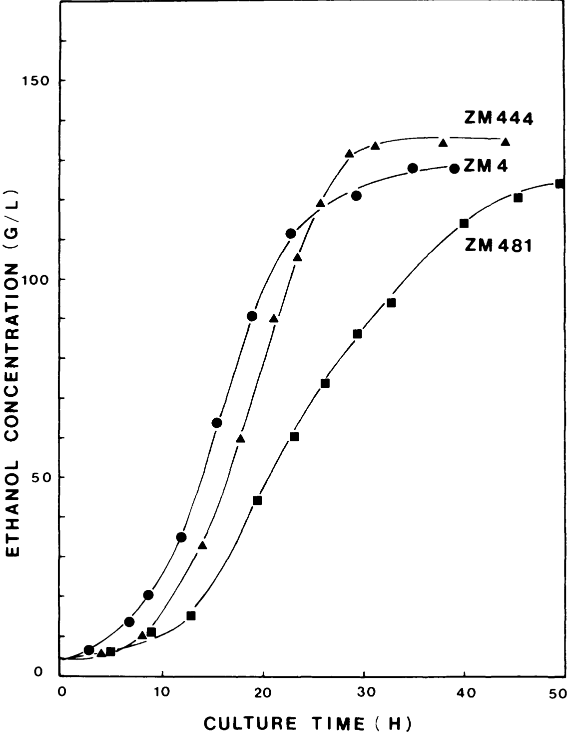

Figure 4. Ethanol production with 300g/1 glucose in the fermenter.

would completely inhibit growth of the parent strain. Of the few colonies which grew on these plates, one (ZM482) was able to grow and produce ethanol almost twice as fast as the parent strain on molasses, both in test tubes and in the fermenter (Figure 6).

Selection for Temperature Tolerance

Temperature is likely to play an important role in the optimal control of ethanol fermentations (30), particularly where cell recycle, vacuum operation or simultaneous saccharification and fermentation are contemplated. Therefore, if the optimum temperature for Zymomonas could be increased from 30°C, the process could be more economical overall. Viikari et al.(32) found that their strains did not grow above 37°C, and ethanol production decreased markedly above this temperature although it did not completely cease even at 45°C. The strains studied in this laboratory also show reduced levels of growth at or above 37°C, although ZM4 proved to be the most temperature tolerant with limited growth and ethanol production at 42°C. However it was fairly easy to select mutant colonies which grew on

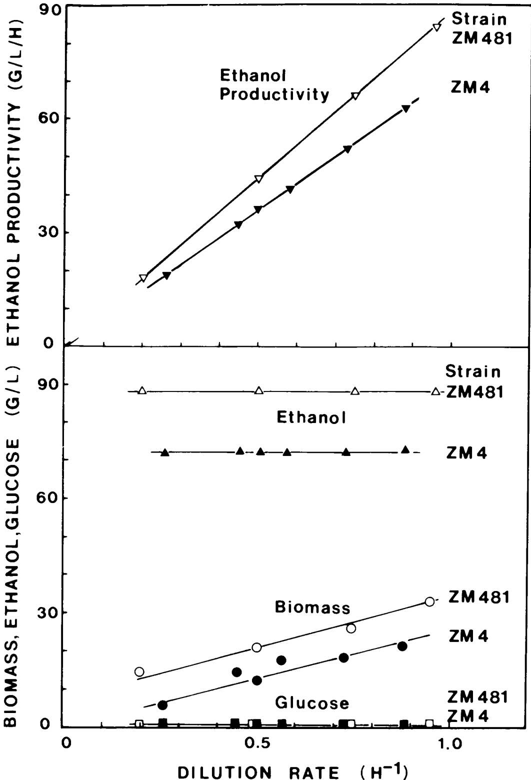

Figure 5. Steady state data in continuous culture with cell recycling for ZM4 (15% glucose medium) and ZM481 (18% glucose medium).

plates at 40°C and above. These mutants grew more readily in liquid media at the elevated temperatures, although increases of only a few degrees have so far been obtained.

DEVELOPMENT OF OTHER GENETIC TECHNIQUES IN ZYMOMONAS

Isolation of Auxotrophs

In order to facilitate strain construction and genetic mapping, a bank of auxotrophic mutants is currently being isolated in this laboratory. It was first necessary to develop a minimal medium for isolation of such mutants, since no suitable medium was available (most experiments reported on Zymomonas have used rich media containing yeast extract). Mutants have been isolated after NTG mutagenesis, and already a number of single and double auxotrophs with various combinations of spontaneous antibiotic resistance markers have been characterized.

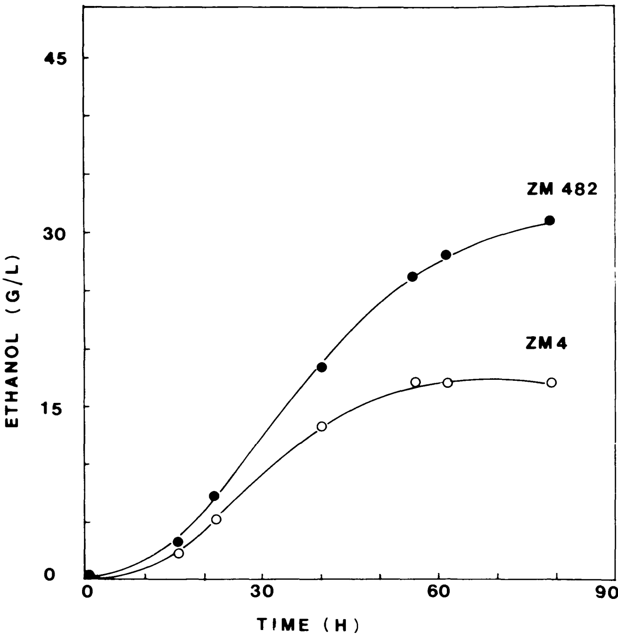

Figure 6. Ethanol production from 20% v/v molasses by strains ZM4 and ZM482 in test tubes.

Conjugation

The transfer of several conjugable drug resistance plasmids of the IncPI and IncFII groups into Z. mobilis has been tested in this laboratory using the membrane filter mating technique (29). All four of the plasmids which were tested were transferred from E. coli and P. aeruginosa to strains ZM1, ZM4 and ZM6 at the high frequencies of 10^{-4} to 10^{-1} (Table VI). Plasmid R68.45 was transferred most readily, with about 30% of the recipient cells gaining the plasmid (Table VI).

When Z. mobilis strains carrying any of the four plasmids were used as donors in conjugation experiments with other marked strains of Z. mobilis, these plasmids could be transferred at very high frequencies (Table VII). As would be expected for such plasmids (29), the transfer between Z. mobilis strains was considerably higher than for the intergeneric matings. However transfer between homogenic strains was no higher than between strains of completely different origins (Table VII).

TABLE VI
Transfer of Plasmids from *E. coli* and *P. aeruginosa* to *Z. mobilis*

Donor*	Recipient*	Approx. frequency of plasmid transfer
E. coli (R1-*drd*-19)	ZM1	$5x10^{-2}$
	ZM6	$1x10^{-2}$
E. coli (pJB4JI)	ZM1	$4x10^{-4}$
	ZM4	$6x10^{-4}$
	ZM6	$9x10^{-3}$
E. coli (pRD1)	ZM1	$1x10^{-2}$
	ZM4	$2x10^{-3}$
	ZM6	$4x10^{-3}$
P. aeruginosa (R68.45)	ZM1	$4x10^{-1}$

*Donor strains were easily distinguishable from the recipient strains morphologically, and usually doubly marked antibiotic-resistant recipient strains were also used.

TABLE VII
Retransfer of R Plasmids between *Z. mobilis* Strains

Donor*	Recipient*	Approx. frequency of plasmid transfer
ZM1 (pRD1)	ZM6	$8x10^{-1}$
ZM4(pRD1)	ZM6	$9x10^{-1}$
ZM6(pRD1)	ZM6	$5x10^{-1}$
ZM4(pJB4JI)	ZM1	$8x10^{-1}$
ZM1(R1-*drd*-19)	ZM6	$8x10^{-1}$
ZM1(R68.45)	ZM6	$8x10^{-1}$

*Donor and recipient strains used were usually doubly marked antibiotic-resistant strains so that they could be easily distinguished from each other.

Chromosome Mobilization

When one spontaneous rifampicin-resistant strain of *Z. mobilis* containing plasmid R68.45 was used as the donor in a conjugation experiment with another marked strain of *Z.mobilis*, it was possible to test for mobilization of the chromosomal rifampicin resistance. A low level of transfer of this gene was detected in a filter mating, at a frequency of almost 10^{-6} which is 100 times higher than the background level of spontaneous mutation (29).

Some of the auxotrophic mutants isolated in the background of ZM4 were also used in tests for chromosome mobilization by plasmid R68.45. Depending on the particular mutant used, levels of transfer ranged from 10^{-5} to more than 10^{-3}.

Since chromosome transfer by this method has now been established for *Z. mobilis*, plasmid R68.45 is currently being used to construct strains more useful for ethanol production, such as a flocculant, highly ethanol-tolerant strain from donor and recipient strains ZM401 and ZM481.

Plasmid Isolation

The utilization of sucrose by *Zymomonas* has been reported by some authors to be an inducible, strain-specific phenomenon (7,9, 15,24,31). A product formed by most strains which do utilize sucrose is levan, a fructose polymer (7,9). However levan production disappears with increasing temperature (30), and one strain, Ag11, does not produce any detectable levan when growing on sucrose (30).

To test whether the inducibility of growth on sucrose and levan production might be correlated with plasmid-borne genes, and their possible amplification in the presence of sucrose, a method was developed for the isolation of natural *Zymomonas* plasmids.

The method used is useful for any volume of culture between 0.2 and 10ml, and could be scaled up further. It is based on the alkaline extraction method of Birnboim and Doly (5), with several modifications to allow isolation of good plasmid preparations with as little chromosomal DNA contamination as possible. Other method involving lysis with Triton X-100 or phenol/chloroform extractions sheared all the DNA very easily, as did the unmodified alkaline extraction method. However, after increasing the buffering strength of the lysing solution, halving the amount of alkali, and reducing the time for coagulation and precipitation of chromosomal DNA and protein, good preparations were obtained with plasmids which were clearly visible on 1% agarose gels. It was also important to use only dilute suspensions of log-phase cells to obtain clear preparations, and this method is unsuitable for direct plasmid isolation from colonies.

Twelve different strains of Zymomonas (as listed in Table II) were grown in glucose medium and tested for the presence of natural plasmids. Interestingly, the most widely used strain (ZM1), which has been maintained in various laboratories for a longer time than the other strains, was the only one with no detectable plasmids (Figure 7).

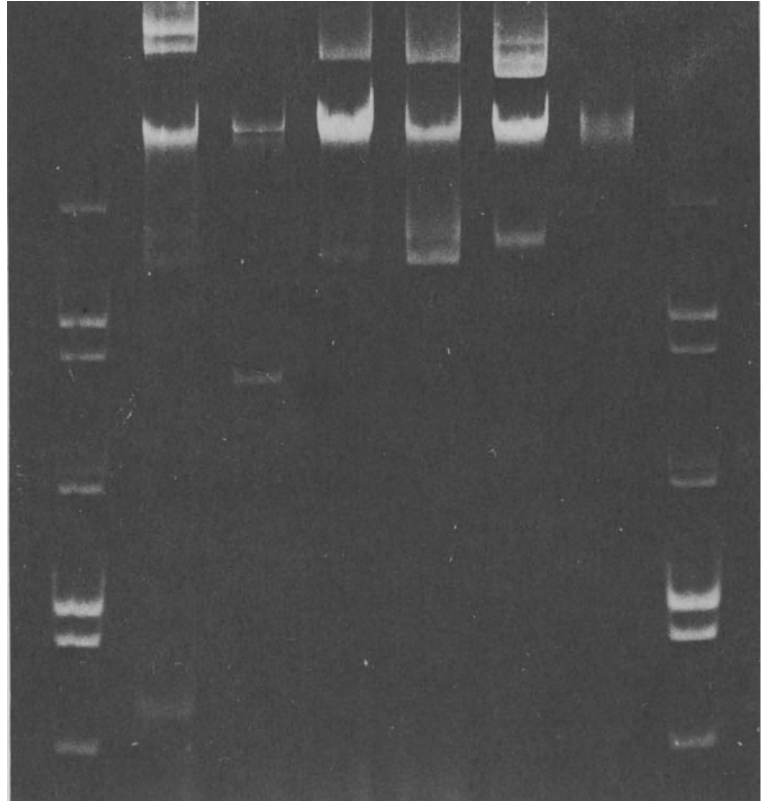

Figure 7a. Plasmids of Z. mobilis on a 1% agarose gel. From left, plasmid standards from E. coli V517; B70; Ag11; ATCC 29192; ZM6; ZM4; ZM1; E. coli V517 (21).

All strains other than ZM1 had at least one plasmid, and usually several. Plasmids ranged in size from 2.5 megadaltons to about 100 megadaltons, and plasmids at both ends of the size range could be found in the same strains (for example, strains 238, Za10 and B70). Another interesting point is that plasmids of apparently the same sizes occurred in strains from widely differing origins (for example, strains ZM6 from fermenting Elaeis sap and B70 from British ale both had plasmids of 15.3 and 18.4 megadaltons). Whether these

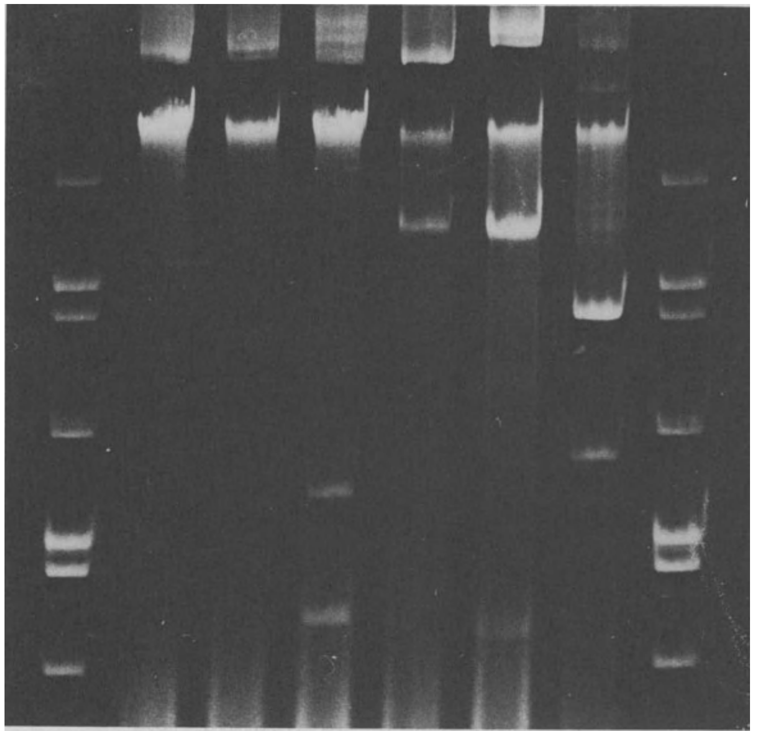

Figure 7b. Plasmids of *Z. mobilis* on a 1% agarose gel. From left, *E. coli* V517; S30.2; S30.A; 238; ZAbi; Za10; 3TH Delft; *E. coli* V517.

plasmids are, in fact, identical is currently being investigated using restriction endonucleases as well as by more accurate sizing. Another possibility being investigated is whether the very small plasmids (2.5 to 4 megadaltons in size) might be useful as cloning vehicles in this organism.

It was not possible to demonstrate any correlation between growth on sucrose and an alteration in the plasmid profiles of any of the levan-producing strains examined. Also, strain ZM1 which had no detectable plasmids, was able to grow on sucrose just as well as the other strains. Therefore, at this stage there is no evidence for any connection between the inducibility of growth on sucrose and the presence of amplification of plasmids.

The situation may not be quite so clear in the case of strain Ag11, which does not produce any levan and which takes longer than most of the other strains to initiate growth on sucrose. In this strain, there is some preliminary evidence which indicates that one

plasmid may be lost fairly readily during growth on glucose but not sucrose. However further investigations are necessary to confirm that this plasmid is really connected with growth on sucrose.

FURTHER IMPROVEMENT OF *ZYMOMONAS*

Strain ZM481 was selected as the most ethanol-tolerant mutant from two sequential mutagenesis experiments on strains ZM4 and ZM48. No success was obtained in attempts to further improve this tolerance by a third round of mutation; no colonies appeared on plates with ethanol concentrations higher than 15% v/v. It may, however, be possible to select better mutants of ZM481 on the basis of greater viability in liquid media with high ethanol concentrations, since this appears to be the main advantage ZM481 has over ZM4.

Similarly, strains with better viability at 42°C and above, and also in molasses, may give higher productivities under these particular conditions. Another factor which should help to increase the yield on molasses is the abolition of levan formation. Although it has not been possible to isolate mutants of strains ZM1 or ZM4 which do not produce levan from sucrose, the availability of the non-levan producing strain Ag11 should facilitate genetic construction of a ZM4 derivative to give higher ethanol yields on sucrose-based substrates.

In order to help with locating the genes for such functions as levan production, as well as for other mutagenesis experiments, a method of transposon mutagenesis is being developed in *Z. mobilis*. Although the Tn5-containing plasmid pJB4JI, which has been used for transposon mutagenesis in Rhizobium (4) is stable in *Z. mobilis* (29), the subsequent introduction of plasmid pJB3JI (a kanamycin-sensitive derivative of R68.45) promotes "jumping" of the transposon and loss of pJB4JI in derivatives of strain ZM4. Such a method may also prove useful for putting markers on some of the natural *Z. mobilis* plasmids.

Z. mobilis does have one disadvantage if it is to be used industrially, due to the fact that it can only use glucose, fructose and sucrose, and cannot utilize other carbon sources such as maltose and starch (31). Since some of the substrates proposed for industrial ethanol production are basically starch (e.g., corn, cassava and wheat), it would be very useful to have a strain of *Z. mobilis* which could use starch. This would then allow direct fermentation of starch to ethanol by *Z. mobilis*, rather than a more costly hydrolysis followed by fermentation. In high productivity systems, where high rates of starch hydrolysis will have to be achieved, the cost of using a commercial glucoamylase enzyme would be a significant factor in the process.

Research into improving Z. mobilis strains and continuous culture systems has shown that high productivities (in excess of 100g/l/h) at ethanol levels of 80-90g/l can be achieved. These values are vastly better than for traditional yeast processes, and it is clear that future significant developments with the Zymomonas process will depend on the construction, by recombinant DNA techniques, of Zymomonas strains with the ability to ferment starch and cellulose-based substrates directly to ethanol.

ACKNOWLEDGEMENTS

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DISCUSSION

Q: CHASE: Since you blame the problems of molasses on Mg^{++} and K^+, could you comment on Doelle's claim that high Mg^{++} is good for Zymomonas?

A: SKOTNICKI: Yes, it is, at 5 g $MgSO_4$/liter. We think there are much higher levels of Mg^{++} and K^+ in molasses.

Q: CHASE: Has anyone looked to see whether the Mg^{++} or the $SO_4^=$ is responsible? Do other Mg^{++} salts do as well? (refers to positive effect at g/l).

A: SKOTNICKI: Someone in our lab is looking at this and has concluded that Mg^{++} and K^+ are the worst ions for inhibiting growth at the levels present in molasses. $MgCl_2$ is as good as $MgSO_4$.

Q: STOKES: Those plasmids that are transferred in from E. coli and P. aeruginosa. Do you know if they are stable once transferred into Zymomonas?

A: SKOTNICKI: Yes, we have found these plasmids to be stable in Zymomonas, except that in a very recent experiment where we looked at chromosome transfer by one of them we did see some segregation.

STRUCTURE AND EXPRESSION OF YEAST GLYCOLYTIC GENES

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INTRODUCTION

Yeast glycolytic enzymes are present at high intracellular concentration and comprise 25-65% of the soluble cellular protein (1). The properties of the isolated enzymes have been studied extensively and in some cases the primary structure of the enzymes has been determined as has the three dimensional structure. The intracellular concentration of many glycolytic enzymes increase when cells are shifted from growth on a nonfermentable carbon source to growth on glucose. The kinetics of this increase in enzyme specific activity has been studied and the data suggest that expression of the glycolytic genes may be coordinated (2,3). Coordinate expression of glycolytic genes is also suggested by the phenotype of a mutant yeast strain which fails to synthesize most if not all of the glycolytic enzymes (4).

We have isolated the yeast genes which encode glyceraldehyde-3-phosphate dehydrogenase and enolase and have investigated the expression of these genes in order to determine the mechanisms involved in controlling the synthesis of these two glycolytic enzymes. The mRNAs which encode glyceraldehyde-3-phosphate dehydrogenase and enolase have been isolated and identified. Utilizing hybridization probes synthesized from the fractionated mRNAs, the genomic structural genes were isolated on bacterial plasmids. The primary structures of three nontandemly repeated glyceraldehyde-3-phosphate dehydrogenase genes and two nontandemly repeated enolase genes have been determined. Several novel structural features of these genes have been identified which may be involved in determining their transcriptional properties. The expression of the two enolase genes has been studied in wild type cells and a mutant containing a targeted

deletion of one of the enolase genes. The data demonstrate that both enolase genes are expressed and that the amount of enolase synthesized from each gene is highly dependent on the carbon source used to propagate the cells.

ISOLATION AND IDENTIFICATION OF YEAST GLYCOLYTIC MESSENGER RNAS

Yeast RNA directs the synthesis of a number of major polypeptides in cell-free extracts. Several of these major polypeptides have been identified on the basis of coelectrophoresis with purified glycolytic enzyme markers and in some cases by selective precipitation with antibody directed against a specific glycolytic enzyme(5). Abundant poly(A)-containing mRNAs, isolated by preparative polyacrylamide gel electrophoresis, direct the synthesis, *in vitro*, of the same major polypeptides. Several of these polypeptides have been identified by tryptic peptide mapping (6).

Two of the most abundant yeast poly(A)-containing mRNAs encode glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and enolase (6). These mRNAs were rigorously identified by tryptic peptide mapping of the polypeptide synthesized *in vitro* under the direction of each mRNA. Since the partially purified mRNAs contained many contaminating nonglycolytic mRNAs, it was necessary to analyze the tryptic peptides under conditions which emphasized those peptides which were synthesized from the abundant mRNA in the population. The fractionated mRNAs were translated in a wheat germ cell-free extract in the presence of ^{3}H-arginine and ^{3}H-lysine. Since each labeled tryptic peptide derived from the translation reaction contained a single arginine or lysine residue, it was possible to identify those peptides derived from the major polypeptide synthesized *in vitro*. An excess of purified GAPDH or enolase was added to the mixture of labeled polypeptides prior to digestion with trypsin. Tryptic peptides were resolved by reverse phase high pressure liquid chromatography. Marker peptides derived from the purified enzyme were monitored spectrophotometrically at 215nm. Labeled peptides were monitored by determining the amount of ^{3}H-peptide in fractions collected from the column. An analysis of the polypeptide synthesized under the direction of fractionated GAPDH mRNA is shown in Figure 1. Panels A and B are chromatograms of peptides derived from polypeptides synthesized *in vitro* in the presence of ^{3}H-arginine and ^{3}H-lysine respectively. In all cases the labeled peptides cochromatograph with GAPDH marker peptides. Panel C is a chromatogram of peptides derived from polypeptide synthesized in the presence of ^{3}H-argine and ^{3}H-lysine. Again, all the labeled peptides cochromatograph with the marker peptides. These data confirm that the major polypeptide synthesized in response to the fractionated mRNA is GAPDH. Since there is a one to one correspondence between the labeled and marker peptides, it is unlikely that the fractionated mRNA contains a second major mRNA contaminant.

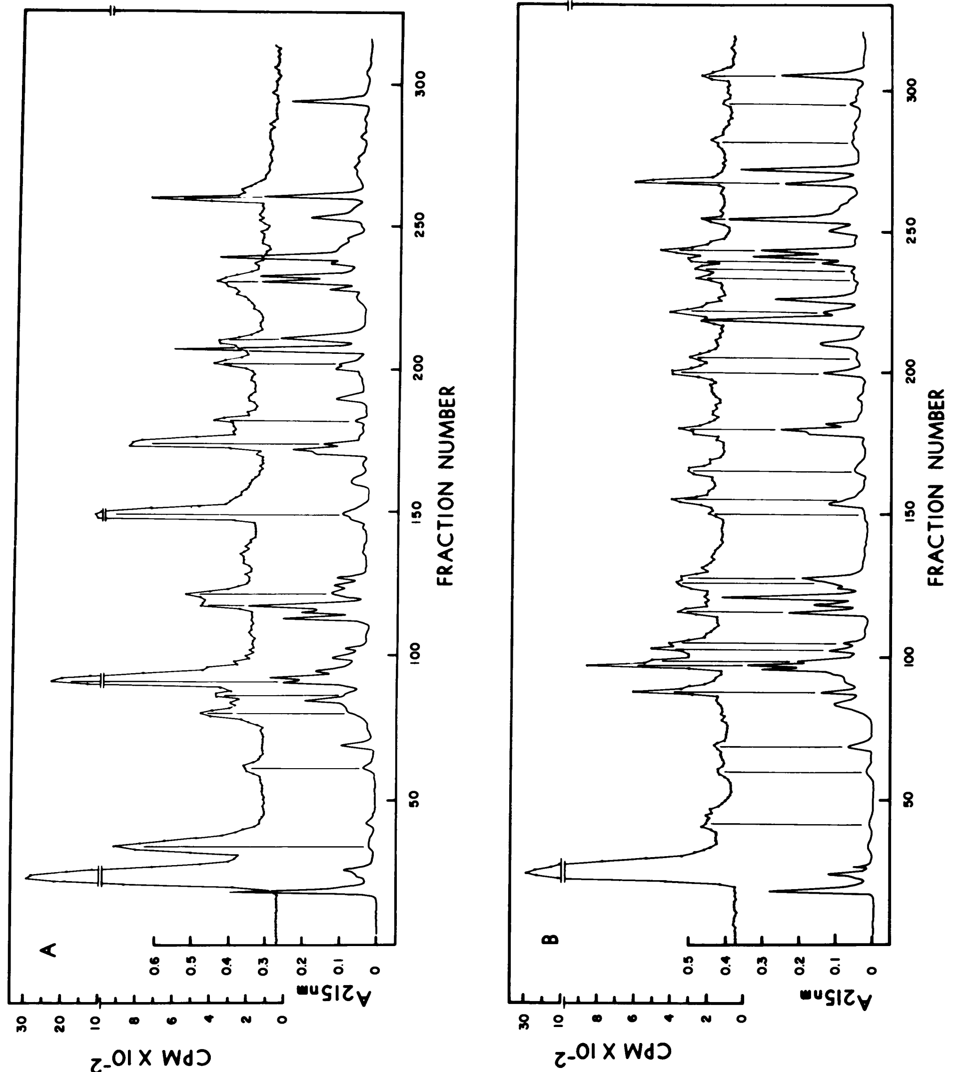
It is not possible to estimate the purity of the fractionated mRNA from the tryptic peptide analysis. The purity of the fractionated GAPDH and enolase mRNAs was estimated from the kinetics of hybridization of the fractionated mRNA with a labeled cDNA probe synthesized from the fractionated mRNA. For GAPDH and enolase, approximately 25% of the cDNA rapidly formed hybrids with the mRNA.

ISOLATION OF YEAST GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND ENOLASE GENES ON BACTERIAL PLASMIDS

In order the determine the apparent number of GAPDH and enclose genes in yeast, Southern blots of yeast DNA, digested with a number of restriction endonucleases, were probed with labeled cDNA synthesized from the fractionated mRNAs. The analysis suggested that yeast contain three GAPDH structural genes and two enolase genes. All five structural genes were subsequently isolated on bacterial plasmids utilizing subculture cloning methods (7-9). The location of the structural gene in each isolated hybrid plasmid was determined by restriction endonuclease and transcriptional mapping. The DNA sequence of a portion of the coding region of each isolated gene was determined. In each case the sequence of the polypeptide predicted from one reading frame of the DNA sequence corresponded to a portion of the known primary structure of either GAPDH (10) or enolase (11). Based on Southern blotting analysis of yeast DNA digested with a number of restriction endonucleases and the restriction maps of the isolated structural genes, none of the GAPDH or enolase genes are tandemly linked.

THE PRIMARY STRUCTURES OF THE YEAST GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND ENOLASE GENES

The complete nucleotide sequences of the GAPDH and enolase genes were determined in order to define more clearly the structural relationships among the gene repeats and to determine if there were any similarities between the GAPDH and enolase genes. The nucleotide sequences demonstrated that all of the genes contain continuous reading frames and translational initiation and termination codons. None of the genes contains an intervening sequence. The GAPDH genes contained within the hybrid plasmids designated $\underline{pgap}^{49}$ and $\underline{pgap}^{63}$ are 94% homologous and the polypeptides encoded by these genes differ in 15 of 331 amino acids (12,13). The GAPDH gene contained in the hybrid plasmid designated $\underline{pgap}$11 is 85% homologous to the other two genes. This divergence pattern suggests that there were two duplication events which gave rise to the three gene repeats in the cell. The gene contained in $\underline{pgap}$11 is probably a product of the first duplication event and the others are the products of a subsequent duplication. Based on the divergence rate


A
B
CPM X 10⁻²
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FRACTION NUMBER
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A215nm

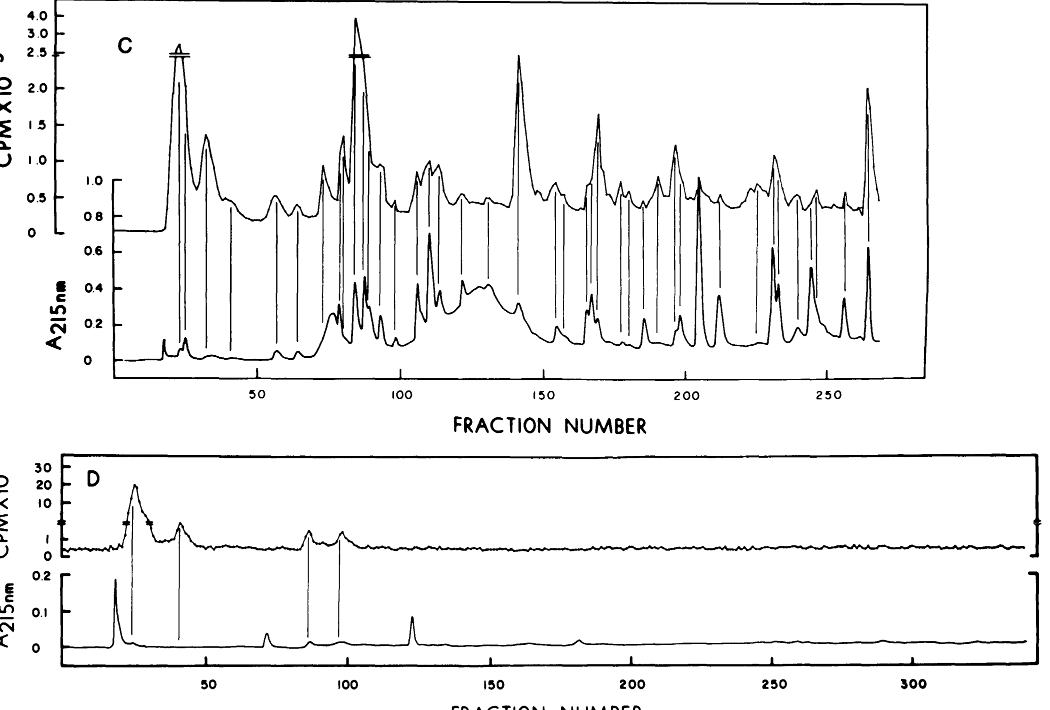

Figure 1. Reverse phase high pressure liquid chromatograms of typtic peptides derived from ^{3}H-labeled polypeptides synthesized in a wheat germ cell-free extract under the direction of yeast GAPDH mRNA. A mixture of purified yeast GAPDH and ^{3}H-labeled polypeptides synthesized _in vitro_ from partially purified GAPDH mRNA was digested with trypsin and the peptides were resolved by reverse phase HPLC. Chromatography was carried out with a C18 reverse phase column and the peptides were eluted with a linear 0-50% gradient of acetonitrile in 10mM sodium phosphate buffer pH 5.8. Unlabled marker peptides were monitored spectrophotometrically at 215nm and ^{3}H-labeled peptides were monitored by scintillation counting of fractions eluted from the column. Panel A: a chromatograph of tryptic peptides derived from polypeptides synthesized _in vitro_ in the presence of ^{3}H-arginine. Panel B: a chromatogram of tryptic peptides derived from polypeptides synthesized in the presence of ^{3}H-lysine. Panel C: a chromatogram of ^{3}H-arginine and ^{3}H-lysine. Panel D: a chromatogram of tryptic peptides derived from the wheat germ cell-free extract in the presence of both labeled amino acids but in the absence of GAPDH mRNA and GAPDH marker protein.

TABLE I

Codon Utilization in Graph and Enolase Genes

Codon	Count	Codon	Count	Codon	Count	Codon	Count
Ala GCA	1	Arg CGA	0	Asn AAT	2	Asp GAT	41
T	172	T	2	C	73	C	89
C	41	C	0				
G	0	G	0				
		AGA	59				
		G	0				
Cys TGT	8	Gln CAA	36	Glu GAA	92	Gly GGA	0
C	0	G	0	G	1	T	147
						C	0
						G	0
His CAT	2	Ileu ATA	0	Leu TTA	5	Lys AAA	15
C	42	T	49	G	133	G	137
		C	56	CTA	2		
				T	0		
				C	0		
				G	0		
Met ATG	33	Phe TTT	5	Pro CCA	62	Ser TCA	0
		C	56	T	2	T	62
				C	0	C	72
				G	0	G	0
						ATG	0
						C	0
Thr ACA	0	Trp TGG	19	Tyr TAT	3	Val GTA	0
T	51			C	48	T	95
C	59					C	79
G	0					G	0

of GAPDH (14) (1% sequence change per 20 million years), minimum estimates of the time of these duplication events are approximately 300 million years ago and 100 million years ago respectively. These are minimum estimates since the possibility that these genes undergo concerted evolution cannot be ruled out. The two enolase genes are 95% homologous and the polypeptides encoded by the genes differ in 20 of 436 amino acid residues (9).

Based on the known amino acid sequences of yeast GAPDH (10) and enolase (11), it is possible to identify the structural gene which encodes the polypeptide that was sequenced. In the case of GAPDH, the sequence predicted from the gene contained in p*gap*49 agreed with the reported amino acid sequence in all but two positions. Interestingly, high molar yields of two amino acids were reported at these positions. One of the amino acids agrees with that predicted from the p*gap*49 sequence and the others agree with the amino acids predicted at these positions from the sequences of p*gap*63 and p*gap*11. These observations suggest that a mixture of at least two GAPDH polypeptides was sequenced and that the polypeptide encoded by the *gap*49 structural gene is probably the most abundant form of the enzyme in baker's yeast (baker's yeast GAPDH was sequenced by Jones and Harris (10)). These data also suggest that at least two GAPDH structural genes are expressed. In the case of enolase, the amino acid sequence predicted from the gene contained in p*eno*46 is in close agreement with the amino acid sequence of an abundant form of enolase isolated from baker's yeast (11). Evidence is presented in a following section that both enolase genes are expressed.

The GAPDH and enolase genes follow an extremely biased codon usage pattern (Table I). With one exception, alanine, aspartic acid

isoleucine, serine, threonine and valine are encoded by two codons containing either U or C in third position. The remaining 14 amino acids are encoded by a single codon in greater than 98% of the cases. The codons represented in this biased pattern are the most abundant codons found in a number of yeast structural genes. The incidence of exceptions to this pattern is substantially higher in the yeast genes which code for cytochrome c (15) and actin (16), however. Abundant isoaccepting +RNAs have been reported for most of the codons represented in the biased pattern and it is possible that the bias is related in some way to the high abundance of these +RNAs. There are some features of the bias which are not explained on the basis of +RNA abundance. In the cases of the six amino acids which are encoded by two codons, there is less than 10% of the predicted draft at third position of the codons which specify amino acids which are conserved in the gene repeats. This feature of the codons distribution suggests the possibility that a specific context has evolved within the coding regions of these genes and maintenance of this context is at least partially for the conservative use of codons in the genes.

The 5' noncoding sequences adjacent to the translational initiation codons of the GAPDH and enolase genes have been analyzed for the presence of sequences which may be involved in modulating expression of the genes and for homologies among the gene repeats. In all cases the A+T composition of the sequences approximately 150 nucleotides upstream from the initiation codon is approximately 80%. The nucleotide sequences 100 nucleotides upstream of the initiation codons in gap49 and gap63 are 70% homologous. Sequence homology in this region is also present in the gap11 structural gene (13). The encolase genes contained in peno8 and peno46 are also homologous within the first 100 nucleotides adjacent to the initiation codons (9). All three GAPDH genes and the eno46 gene contain a TATAAA sequence approximately 130-160 nucleotides upstream from the coding sequences. This sequence is thought to play a role in regulating transcription of eucaryotic genes (17). This sequence is absent in the eno8 gene, however, there are sequences similar to TATAAA in the same region of the noncoding sequences of this gene.

The most striking feature of the 5' noncoding regions of the GAPDH and enolase genes is the fact that all five genes share extensive nucleotide sequence homology within the first 50 nucleotides upstream from the translational initiation codons. The nucleotide sequences in these regions are shown in Figure 2. All five genes contain a CACACA sequence 6-15 nucleotides upstream from the initiation codons. The genes also contain a 15 nucleotide sequence 22-41 nucleotides upstream from the initiation codons which is 90% conserved among the gene repeats. Interestingly, this conserved region has been duplicated in the 5' noncoding region of the gap11 structural gene. Since the GAPDH and enolase genes

```
        (-72)CAAG-AACTTGGTTT-GATATTTCACCAACACACAC(-39)
(-38)AAAAAAC--AG-TACT-----TCA--CTAAATTTA-CACACA--------AAACAAA ATG   pGAP11
(-47)AAAACACCAAG-AACTTAGTTTCGAAT-AAA-----CACACATAAA--TAAA-CAAA ATG   pGAP491
(-56)AAAAAACCAAG-AACTTAGTTTCAAATTAAATTCATCACACAAACAAACAAAACAAA ATG   pGAP63
(-45)AAAAAACCAAGCAACT--GCTT---ATC-AA-----CACACAAACA-CTAAATCAAA ATG   pENO46
(-38)AATAACACCAAGCAACTAATACT---AT--AA-----CATACAA-----TAA-T---A ATG   pENO8
```

Figure 2. The nucleotide sequences of the 5' noncoding regions of the three GAPDH genes and the two enolase genes adjacent to the translational initiation codons in each gene. The numbers in parentheses refer to the number of nucleotides upstream from each initiation codon. The brackets below the peno8 sequences indicate the location of two highly conserved regions in the five sequences.

have not evolved from a common precursor to the coding regions of the genes it is significant that the 5' noncoding regions of these genes are homologous. It is likely that these homologous sequences did evolve from a common precursor suggesting that the coding regions of GAPDH and enolase recombined adjacent to a common nucleotide sequence at some point in their evolution. There are a number of short (8-16 nucleotides) direct nucleotide sequence repeats within the 5' and 3' noncoding regions of the GAPDH and enolase genes (9,13). It is possible that these repeated sequences are remnants of recombinational events which resulted in the fusion of these glycolytic coding sequences to noncoding sequences which may regulate their expression.

Comparison of the 3' noncoding sequences of the GAPDH and enolase genes showed that these sequences are approximately 80% A+T within 100-200 nucleotides downstream from the translational termination codons (9,13). The GAPDH genes contained in pgap49 and pgap63 are 70% homologous within 100 nucleotides adjacent to their respective termination condons. There is little if any homology in this region of the gap11 gene. The two enolase genes are approximately 70% homologous within 250 nucleotides downstream from their respective termination codons. All five genes contain an AATAA sequence in the 3' noncoding sequences. This sequence is thought to play a role in selection of poly (A) addition sites in eucaryotic mRNAs (18). The AATAA sequences in the GAPDH genes are 40-60 nucleotides downstream from the respective termination codons. This sequence in the enolase genes is approximately 250 nucleotides downstream from the respective termination codons. Comparison of the 3' noncoding sequences in GAPDH and enolase revealed little if any nucleotide sequence homology.

The possibility that there are repeated (hybridizable) sequences adjacent to the GAPDH and enolase genes was tested. Southern blots of yeast genomic DNA, digested with a variety of restriction

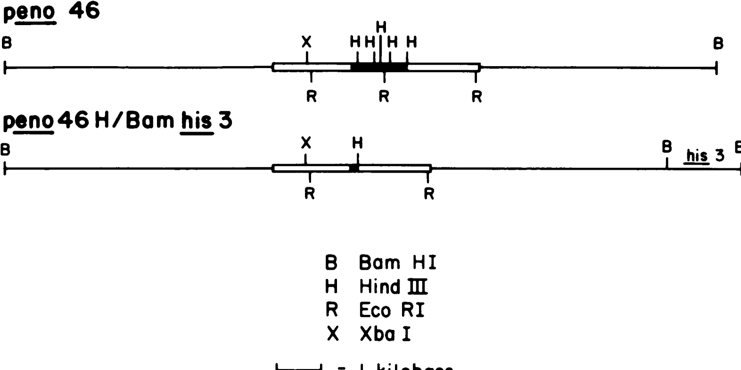

Figure 3. The restriction endonuclease cleavage maps of peno46 and a hybrid plasmid derived from peno46 which contains a deletion of 90% of the enolase coding sequences and a functional his3 structural gene. The single horizontal line indicates the location of p2124 plasmid vector sequences. The double line represents a cloned segment of yeast genomic DNA which contains the eno46 structural gene. The blacked out region indicates the location of the enolase coding sequences.

endonucleases, were probed with nick translated restriction fragments derived from the sequences which flank the five structural genes. In all cases these probes hybridized with unique segments of genomic DNA. Based on those dates, the sequences within approximately 5 kilobase pairs of the GAPDH and enolase genes are unique.

COORDINATE EXPRESSION OF YEAST GLYCOLYTIC GENES

Recently, a mutant was reported which fails to synthesize a number of glycolytic enzymes (4). This mutant strain has been analyzed to determine if the mutation blocks transcription of the glycolytic genes. Total cellular RNA was isolated from the mutant strain and translated in a wheat germ cell-free extract. In contrast to RNA isolated from the wild type parent strain grown under the same conditions, RNA isolated from the mutant fails to direct the synthesis of a number of major glycolytic polypeptides. Total RNA isolated from the wild type and mutant strains was hybridized in solution against nick-translated probes sythesized from restriction fragments derived from the coding sequences of the isolated GAPDH and enolase genes. These data confirmed that GAPDH and enolase mRNA sequences are absent in RNA isolated from the mutant strain (the detection limit was 5% of wild type values). These data do not prove that the glycolytic genes are regulated but they do support the notion that these genes are coordinately expressed. It is of obvious interest to test the possibility that the homologous sequences

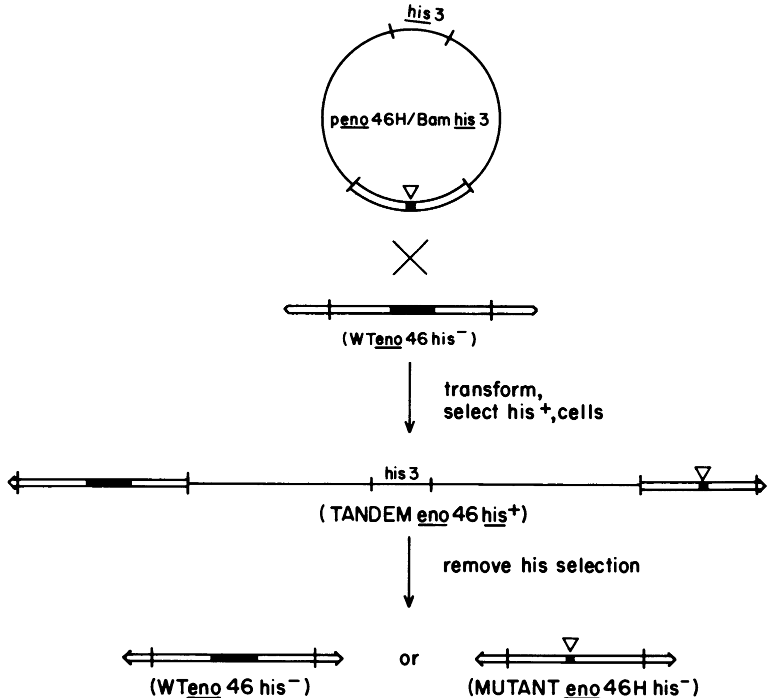

Figure 4. Integrative transformation of a yeast his3⁻ auxotroph with a hybrid plasmid containing a functional his^3 structural gene and a deletion of 90% of the eno46 coding sequences. The structure of the tandem integrative intermediate at the eno46 locus is illustrated as are the structures of the possible products after intramolecular recombination between the repeated sequences in the tandem intermediate.

within the 5' noncoding regions of the GAPDH and enolase genes are involved in the coordination of expression suggested by the mutant phenotype.

EXPRESSION OF THE YEAST ENOLASE GENES

In order to study the expression of the repeated enolase genes, a mutant was constructed which contains a deletion of 90% of the eno46 coding sequences. As described above, the major form of enolase isolated from baker's yeast is encoded by the eno46 structural gene. The eno46 deletion was constructed *in vitro* by removing four Hind 111 fragments from within the coding sequences in the hybrid plasmid peno46. A Bam H1 fragment, containing a functional his3 structural gene, was ligated into a single Bam H1 cleavage site

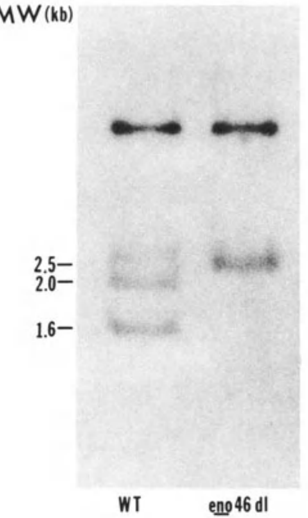

Figure 5. An autoradiogram of a Southern blot of yeast DNA isolated from a wild type strain and a mutant containing a deletion of eno46 coding sequences. DNA was digested with Eco Rl prior to electrophoresis on a 1% agarose gel and transfer to nitrocellulose paper. Hybridization was carried out with a nick-translated probe synthesized from the 2.0 and 1.6kb Eco Rl fragments derived from peno46 which contain the eno46 structural gene. The 2.0 and 1.6kb Eco Rl fragments are present in the wild type DNA and form hybrids with the probe. These fragments are not present in the mutant DNA. A 2.5kb fragment derived from the deletion shown in Figure 3 is present in genomic DNA isolated from the mutant strain.

within the plasmid containing the deletion. The restriction endonuclease cleavage map of this hybrid plasmid is shown in Figure 3. A stable his^- auxotroph was transformed with the plasmid containing the deletion utilizing recently developed methods for yeast transformation (19). His^+ transformants were isolated which had integrated the entire hybrid plasmid at the eno46 locus by homologous recombination. The tandem configuration of the incoming deletion and the wild type resident eno46 gene is unstable. His^+ transformants revert readily to his^- when the cells are propagated in a medium containing histidine. These his^- revertants contain either the resident wild type eno46 structural gene or the incoming deletion. The transformation and subsequent replacement of the resident eno46 gene by the deletion is summarized in Figure 4.

His^- revertants in which the resident eno46 structural gene was replaced by the deletion were identified by Southern blotting

analysis. Genomic DNA was isolated from several his⁻ revertants, digested with Eco R1, electrophoresed on an agarose gel and transfered to nitrocellulose paper. The blot was probed with nick translated Eco R1 fragments isolated from peno46 (2.0kb and 1.6kb) which contain the eno46 structural gene. Revertants which contain the deletion should contain a new 2.5kb Eco R1 fragment derived from the deletion and should not contain the two fragments derived from the wild type gene. As illustrated in Figure 5, the wild type DNA contains the 2.)kb and 1.kb fragments. In contrast, a strain containing the deletion contains only the 2.5kb fragment. Two additional Eco R1 fragments form hybrids with the probe. The large fragment and a fragment which is slightly larger than 2.5kb are derived from the eno8 structural gene and cross hybridize with the probe within the homologous coding sequences of the eno8 structural gene.

The eno46 deletion segregates 2:2 in crosses with a wild type strain. The growth rate of the mutant strain is apparently the same as the wild type strain under a variety of different growth conditions. Wild type strains contain two immunologically precipitable enolase polypeptides which can be resolved on SDS polyacrylamide gels. The mutant strain fails to synthesize one of these polypeptides. Three enolase isozymes were resolved after DEAE Sephadex chromotogrphy. The subunit structures of these isozymes was determined from SDS polyacrylamide gels of the purified isozymes. The subunit structures are consistant with random assortment of the eno8 and eno46 polypeptides into active enolase dimers. As expected, the deletion mutant fails to synthesize two of the three enolase isozymes. Analysis of the enolase isozyme patterns from cells grown on different carbon sources showed that the two genes are differentially expressed. In cells grown on glucose, the eno8 polypeptide is 20 fold more abundant than the eno46 polypeptide. In cells grown on ethanol, the eno46 polypeptide is approximately two fold more abundant than the eno8 polypeptide. The total quantity of enolase in cells grown on ethanol is approximately 20 fold lower than in cells grown on glucose. The most abundant form of enolase in baker's yeast is eno46. Expression of the eno8 gene in wild type cells and the eno46 deletion mutant is quantitatively the same. These data suggest that the two enolase genes are expressed independently. The data also suggest that expression of the enolase genes may be regulated at the transcriptional level.

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DISCUSSION

Q. SWITZER: Are both enolase polypeptides shut down in a mutant which is blocked in a number of glycolitic enzyme activities?

A. HOLLAND: Yes.

FUNCTIONAL MUTANTS OF YEAST ALCOHOL DEHYDROGENASE

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ABSTRACT

Selection of petite strains of yeast (that is, strains unable to respire aerobically) on media containing allyl alcohol will result in enrichment for mutants at the ADC1 locus. This locus codes for the constitutive alcohol dehydrogenase, ADH-I, which is primarily responsible for the production of ethanol in yeast. The mutant enzymes are functional, and confer resistance to allyl alcohol on the cell by shifting the NAD-NADH balance in the direction of NADH. These mutants exhibit altered K_m's for cofactor, substrate, or both, and often have altered V_{max}'s. In this paper, the methodology for obtaining these mutants and for determining the amino acid substitutions responsible for these changes is presented. Several new mutants have been at least approximately localized, and one, D_B-AA3-N15, has been shown to be due to the substitution of an arginine for a tryptophan at position 54. This substitution would be expected, by analogy with the known tertiary structure of the horse liver alcohol dehydrogenase, to decrease the hydrophobic environment of the active site pocket. The substitution has a pronounced effect on the K_m for ethanol, but far less on that for acetaldehyde.

The current status of investigation of other classes of functional mutants of this enzyme, and the potential both for selection of useful variants of this molecule and for an increase understanding of its function are discussed.

INTRODUCTION

There are two major isozymes of yeast alcohol dehydrogenase

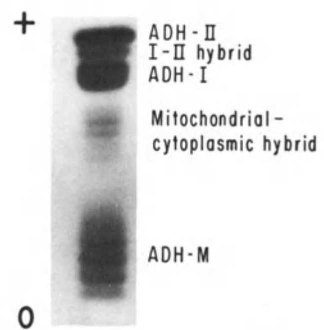

Figure 1. Starch gel electrophoresis followed by activity staining of a crude extract of wild type Saccharomyces cerevisiae. Cells were grown on complete medium with aeration.

(alcohol:NAD oxidoreductase, E.C. 1.1.1.1) in the cytoplasm of yeast cells (19,35). Another isozyme, or possibly more than one, is found in the mitochondria but its appearance in the cell is controlled by a nuclear gene (4). Figure 1 shows the typical pattern seen on starch gel electrophoresis followed by activity staining of a crude extract of yeast cells. Note that the mitochondrial and cytoplasmic isozymes are sufficiently similar that they form weakly staining hybrids. The two cytoplasmic isozymes, however, are sufficiently dissimilar that they form only a single hybrid band even though they are tetramers and might therefore be expected to form a total of five bands. Heterozygotes for two electrophoretically dissimilar forms of one of the cytoplasmic enzymes do in fact exhibit the expected five bands.

In this paper I will discuss methods developed in our laboratory for producing and examining functional mutants of one of the cytoplasmic ADH's. This isozyme, ADH-I, is the slower-moving isozyme at alkaline pH, and is the isozyme chiefly responsible for the production of ethanol under anaerobic conditions. As such, it has considerable economic importance, and the fact that it is so manipulable genetically greatly increases its utility as an experimental system.

The biochemistry of yeast ADH's

The primary sequence of ADH-I was determined by Jornvall (1977), and has since been confirmed except for minor strain differences by Williamson et al. (27 and unpublished results) directly from the DNA sequence. 85% of the sequence of ADH-II has been determined by conventional biochemical means (32), and the two isozymes were found to be 95% identical. The DNA sequence of ADH-II is currently being investigated (Young, pers. comm.).

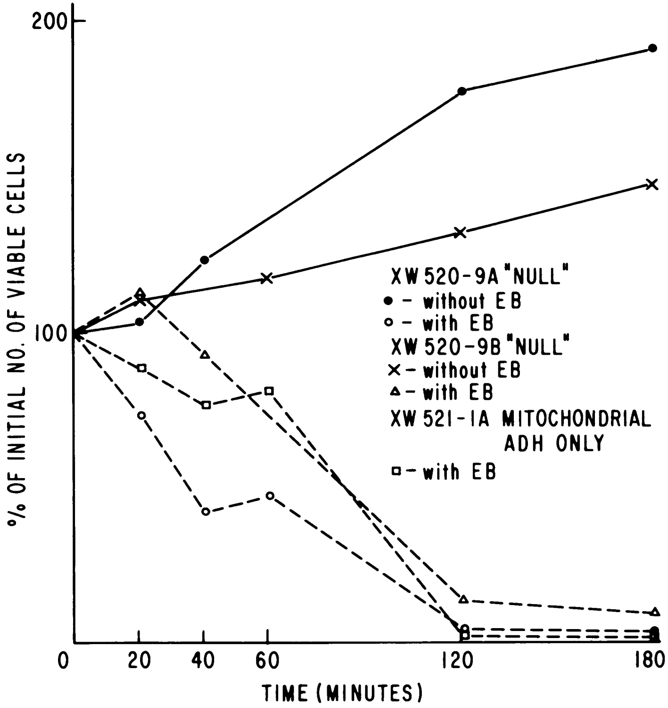

Figure 2. Killing curves produced by ethidium bromide on ADH-deficient cells. Ethidium bromide will kill cells lacking in cytoplasmic ADH activity, whether or not the mitochondrial ADH is present. From Wills and Phelps (35), by permission.

There are considerable differences in the kinetics of the two ADH's (28), which suggest mechanisms by which the two isozymes differ in their preferred direction. The substrate K_m's of ADH-II are considerably smaller than those of ADH-I, particularly the K_m for acetaldehyde, which is 100 times smaller than the acetaldehyde K_m of ADH-I. Since, in an aerobic cell, acetaldehyde is rapidly converted to acetyl CoA, the low K_m for ethanol exhibited by ADH-II would encourage rapid oxidation of ethanol.

The two ADH's also differ biochemically. We were able to separate the two isozymes by affinity chromatography from a single aerobically-grown cell preparation, and showed that ADH-I is almost completely acetylated at the N-terminus, while ADH-II is only 60% acetylated (16). Whether this reflects the physiological state of the cell (the acetyl CoA necessary for N-terminal acetylation may be less plentiful in the cytoplasm of an aerobically grown cell) or whether it provides an indication of different sites and rates of synthesis of the two isozymes remains to be determined.

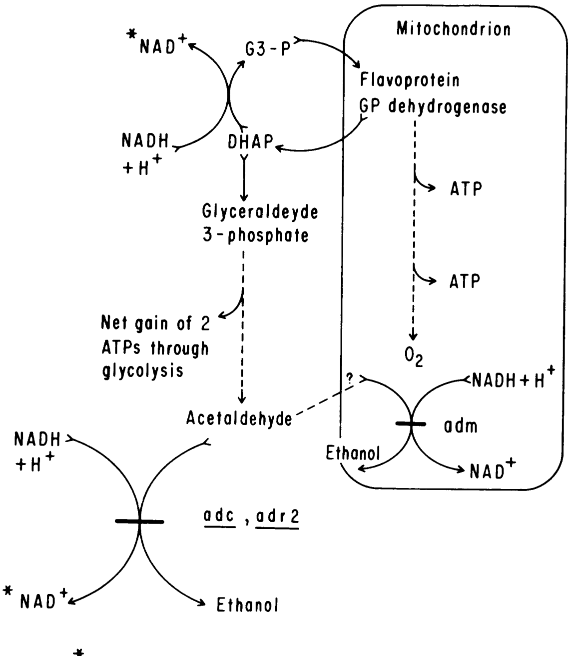

Figure 3. The biochemical pathways affected by blockage of alcohol dehydrogenase activity. Blockage of cytoplasmic activity by mutations in the ADC and ADR2 gene diverts glycolytic activity through the dihydroxyacetone phosphate-glycerol phosphate shuttle. This shuttle, however, can only operate if the mitochondrion is functioning. For further details see text. Modified from Wills and Phelps (35), by permission.

An important aspect of the biochemistry of aldehyde reduction, the final step in anaerobic glucose metabolism in this organism, is shown in Figures 2 and 3 (35). Figure 2 shows the killing curve which results when ADH-negative strains are subjected to ethidium bromide, a curve which is coincident with the production of the petite phenotype in normal cells. Figure 3 shows the biochemical pathways involved in this phenomenon. Alcohol dehydrogenase activity in the cytoplasm is absolutely necessary for the survival of a yeast grown under anaerobic conditions or under conditions where its mitochondria are non-functional. There are two reasons for this.

The first is that without ADH activity normal glycolysis can no longer be completed. Rather, catabolism is almost completely diverted through the dihydroxy-acetone phosphate-glycerol phosphate shuttle, and as a result these cells excrete large amounts of

glycerol. This pathway is made unavailable under anaerobic conditions or when mitochondrial activity is destroyed, and the cell dies as a result. It is not yet certain whether death of the cell is due to a buildup of acetaldehyde in the cell or to starvation. The second reason is rather more complex, and explains why cells in which the mitochondrial ADH is still functioning are also unable to survive as petites even though their mitochondrial enzyme is still active. In the absence of cytoplasmic ADH activity, the only other source of NAD+ in the cytoplasm comes from the dihydroxyacetone phosphate-glycerol phosphate shuttle. This shuttle, shown in Figure 3, transfers reducing equivalents into the mitochondria, where they are used in the production of ATP. Without functioning mitochondria, the NAD+ necessary for glycolysis can no longer be produced, even though acetaldehyde should be perfectly free to diffuse into the mitochondria and be converted to ethanol. This phenomenon, in which cells with functioning mitochondrial ADH die when they are converted to petites, incidentally provides a direct demonstration of the impermeability of the mitochondrial membrane to NAD+.

This phenomenon, first noted by Wills and Phelps (35), provides the basis for both the selection of functional ADH mutants detailed later in this paper and for the cloning of ADH genes. Ciriacy (pers. comm.) improved on the methodology of this selection process by using Antimycin A rather than ethidium bromide. This antibiotic represses electron transport at the level of cytochrome b, and the petite phenocopy thus produced is completely reversible.

The genetics of yeast ADH's

Most of the work on yeast ADH genetics is due to the masterful analysis of Ciriacy (4-7). The structural genes for ADH-I and ADH-II, named adc and adr2 respectively, have been shown to be unlinked.

A series of linked and unlinked modifier genes of the adr2 gene have been isolated, and some of these are of particular interest. Several alleles at a site closely linked to adr2 and named adr3, result in the constitutive production of ADH-II even in petite cells in which this isozyme is normally not made. Some of these alleles have been shown to be due to Tyl-like insertions (8). Another, unlinked locus, adr1, also regulates the production of this isozyme. Recessive mutant alleles at this locus suppress the production of ADH-II, while a series of semidominant alleles named ADR^{C} result in its constitutive production. The ADR1 gene has recently been cloned (C. Denis, pers. comm.), and preliminary results suggest that the product of this gene is synthesized constitutively.

A number of other mutants affecting ADH-II synthesis have been isolated by Ciriacy, including one (CCR1) which affects the synthesis of several enzymes in the glycolytic pathway.

Work by Denis (9) has shown that control of the synthesis of ADH-II is at the level of transcription, and that the ADR1 gene appears to be a positive regulator -- that is, a functioning ADR1 protein is necessary for the production of normal ADH-II mRNA.

Little is known yet about the regulation of the "constitutive" isozyme ADH-I, though we have recently found that it is repressed almost completely by growth on a non-fermentable substrate at high pH (unpublished results). Further, there is no indication yet of the biochemical mechanisms by which either isozyme is regulated, although some tentative suggestions as to mechanism have been made (31).

The production of functional yeast ADH mutants

Although we have recently turned our attention to the biochemistry of yeast ADH regulation, most of the work in our laboratory has concentrated on using the biochemical properties of this system to select for alterations in the structural gene ADC that will change but not destroy the function of the gene product. The selection procedure for producing such mutants is both simple and specific. Since petite cells cannot survive without ADH activity in the cytoplasm (and since ADH-I is the only cytoplasmic isozyme that normally appears in petite cells), we have subjected petite cells to selection in the presence of allyl alcohol. This alcohol (Figure 4) is by itself harmless to yeast cells, but it is readily oxidized by ADH to acrolein, its highly poisonous aldehyde (24). This fact was initially used by Megnet (21) to select for ADH-negative mutants in *Schizosaccaromyces pombe*, and has been used by Ciriacy and ourselves to generate mutants in *Saccharomyces* lacking all three of the isozyme activities. Allyl alcohol selection in petite cells, however, results in a very different kind of pressure. The cell which survives cannot do so because of a lack of ADH activity, since as we have seen this will be lethal. It must survive as a result of a changed property of the cell which leaves normal ADH activity more or less intact. The remarkable thing about this selective procedure is its specificity: approximately a third of the mutants isolated in experiments of this type show altered electrophoretic mobility of the ADH-I band, *prima facie* evidence for an alteration in the structural gene (29). Further, in subsequent generations, the resistance to allyl alcohol co-segregates with the electrophoretic mobility mutants (28). As will be developed later, a very high proportion of the remaining two thirds of the allyl alcohol resistant mutants also carry changes in the structural gene.

Mechanism of allyl alcohol resistance

While the allyl alcohol resistant mutants could be shown to be

located in the structural gene itself, a continuing puzzle for some time was the nature of this resistance, particularly in light of the fact that the ability of the mutant enzymes to oxidize allyl alcohol seemed to be unimpaired. As will be developed in the Results section, the weight of evidence is now that the source of the resistance is a subtle change in the balance of reactants inside the cell. Allyl alcohol itself appears to be harmless to the cell in the absence of alcohol dehydrogenase activity; ADH negative mutants resistant to 10% (v/v) allyl alcohol can be produced readily by repeated cycles of selection (Wills and Pomerantz, unpublished). Further, neither allyl alcohol nor acrolein is metabolized further inside the cell so that the ratio between the harmless alcohol and the harmful aldehyde is a function of the ratio of NAD to NADH as well as of the alcohol and aldehyde. The thermodynamic equilibrium at a given pH, K'_{eq} is given by:

$$K'_{eq} = \frac{[\text{NADH}][\text{Acrolein}]}{[\text{NAD}][\text{Allyl alcohol}]}.$$

The ratio of NADH to NAD is a dynamic function of the cell's metabolism, and can be perturbed by the addition of any substrate acted upon by an NAD-dependent enzyme. It can also be altered by growth under aerobic and anaerobic conditions. ADH activity appears to be an important contributor to this ratio, which, in turn, makes up an important part of the redox balance of the cell. The ratio can readily be perturbed in petite cells by the addition of acetaldehyde or ethanol (34,36). A shift in the ratio in the direction of NADH will, since K'_{eq} is a constant at a particular pH, result in a shift in the ratio of allyl alcohol to acrolein in the reverse direction, towards the harmless alcohol and away from the harmful aldehyde. As will be developed in the Results section, all five of the mutants which have been thoroughly investigated show a shift in the ratio in the direction of NADH which parallels the resistance of the mutants, as does a strain carrying the wild type ADH-II. It seems certain that this shift is due to the small changes seen in the kinetic parameters of the mutant enzymes, although the precise mechanism or mechanisms involved has remained elusive.

Importance of functional yeast ADH mutants

Alcohol dehydrogenases form an important part of a class of dehydrogenases, the evolution and functions of which have undergone intensive investigation (25). Although yeast alcohol dehydrogenase has only about 25% amino acid identity with the thoroughly-investigated horse liver alcohol dehydrogenase there are enough functional homologies to suggest that their tertiary structures are very similar. As will be seen below, evidence from the mutants so far investigated reinforces this impression, in that the effects of

these mutants could be predicted from the probable effects of homologous substitutions in the horse liver enzyme. Thus, an understanding of the mechanisms of the yeast enzyme may have a bearing on the clinically important liver enzyme.

As with other well-studied proteins such as hemoglobin (12) and insulin (3), alcohol dehydrogenase will undoubtedly be discovered to have more than a simple catalytic function in the cell. For example, as will be seen, the two cytoplasmic yeast isozymes play a central role in governing the redox balance inside the cell, and small changes in the kinetics of ADH-I are enough to disturb the redox balance markedly. It is not impossible that the amount and type of ADH present may have an effect on the regulation of important metabolic pathways in the cell.

One major discovery that has emerged from the study of the dehydrogenases is that those which are evolutionarily related (excluding the *Drosophila* ADH, which appears to have been the result of convergent evolution (26)) consist of two halves, a catalytic and a cofactor-binding domain (25). While there is some interaction between the domains, the substrate and cofactor binding sites in yeast ADH appear to exhibit a good deal of autonomy, apparent both from kinetic studies (20,28) and from studies of mutants detailed in this paper and elsewhere (32). As more mutants affecting the binding sites are located, it should be possible to investigate the mechanism of catalysis in great detail.

Finally, the study of functional mutants in this molecule can help to answer evolutionary questions of considerable importance. It is still, for example, quite unclear what proportion of amino acid substitutions that have occurred in natural populations is due to chance and what proportion is due to a selective advantage for the newly-arisen substitution (18,30). One way of casting light on this question is to ask what proportion of a molecule is "seen" by natural selection. That is, what proportion of the residues in a molecule can be changed so as to bring about an alteration in the molecule's function? We are a long way from answering that question, but our preliminary results suggest that the residues affecting function will be found to form a not inconsiderable proportion of the molecular as a whole, and that residues affecting the same function may be widely scattered on the molecule. This is so far consistent with the very extensive data from hemoglobin (22).

METHODOLOGY

Mutant selection and genetics

Functional mutants of yeast ADH have been produced from strain XW517-2D, a cytoplasmic petite (rho minus) with ADH genotype ADC

ADR2 adm. This strain lacks the mitochondrial ADH, and because it is a petite produces only ADH-I. Selection is normally carried out on yeast extract-peptone-dextrose plates containing 5 or 10 mM allyl alcohol, added sterilely after autoclaving. Selection on unmutagenized cells, or on cells mutagenized with nitrosoguanidine or ethyl methane sulfonate (for methods, see 2) has resulted in similar spectra of mutations. Approximately three quarters of the mutants isolated grow badly and have greatly reduced levels of ADH. Of the remainder, about a third show electrophoretic mobility changes in the enzyme, and the majority of these (15 out of 22 in one large series of experiments) migrate more slowly than the wild type. Changes in the structural gene appear to be common among mutants showing no mobility shift, however. Ten mutants showing no mobility change were crossed to an ADH resistance mutant of the opposite mating type which did show a mobility shift. Ten tetrads were examined from each cross, and in every case all the spores gave rise to allyl alcohol resistant strains. This is the pattern to be expected if the genes conferring resistance but no mobility shift were allelic to the known structural gene mutation. It therefore seems very likely that the change leading to resistance is located in the structural genes for these mutants as well, though there is no change in the electrophoretic mobility of these mutant enzymes.

Repeated selection on plates or in a turbidostate (28) results in mutants with enhanced resistance to allyl alcohol, up to 40 mM. One of the mutants isolated from three rounds of selection, XW517-S-AA-1, appears to have two mutational alterations, though the mutational changes have not yet been precisely determined (Wills and Jornvall, unpublished). If this is confirmed, it will be another indication of the great specificity of the allyl alcohol selection procedure.

Selection with crotyl alcohol, a four-carbon alcohol with a double bond, produces a similar spectrum of mutations (unpublished results.

Enzyme purification

Alcohol dehydrogenase makes up approximately 1% of the soluble protein in the yeast cell, and the purification procedure for this enzyme is straightforward, taking one day to yield a preparation giving a single band on an SDS-acrylamid gel. Cells are ground as a paste with 1:1 washed alumina (Sigma Type 305), and the ground slurry is suspended in 100 mM phosphate at pH 7.0 After a preliminary precipitation with protamine sulfate and an acetone precipitation step (23), the supernatant is passed through a column of Cibachron Blue F3GA ("Affi-Gel Blue" from Biorad.) Normally, all the enzyme activity sticks to the column and the enzyme can be

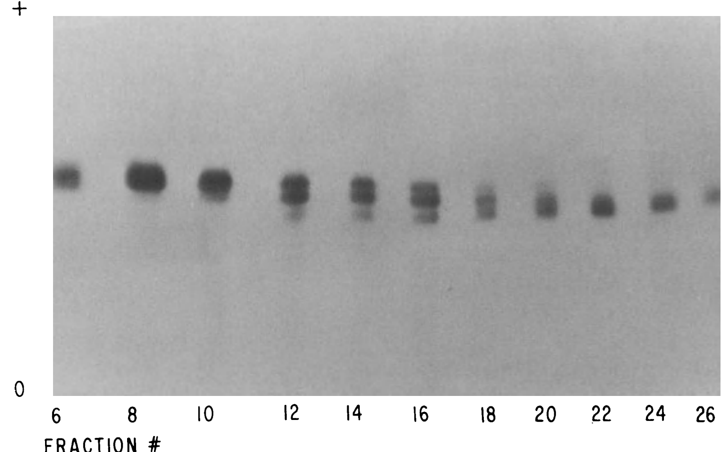

Figure 4. Activity gel showing partial separation of isozymes from a 0-80 μ M NADH gradient. The very strong hybrid band is typical of a diploid strain; haploid strains show a much fainter hybrid band on activity gels.

eluted in pure form with a gradient of 0-80 μ molar NADH which itself has been freshly purified by passage through a DE-11 column. The specificity of this column chromatography procedure is such that the two major isozymes can be partially separated from a cellular preparation which contains both, and the mitochondrial enzyme shows some separation as well (Figures 4 and 5). It was this chromatography technique which allowed us to demonstrate that the two isozymes showed different degrees of N-terminal acetylation.

Occasionally a mutant enzyme will not adhere to the affinity column, and this has been found to be due to its having a very large K_m for substrate or cofactor. Such mutants can be satisfactorily purified on DE11 (DEAE-cellulose) using a gradient from 5-100 mM phosphate at pH 7.0. It may, however, take a second pass through the column using a shallower gradient before the enzyme is sufficiently pure, with inevitable loss of material. Mutant D_B-AA5-N10, discussed in the Results section, is one of this class of mutant.

Enzyme kinetics

Because of the importance of kinetic changes in the mechanism of resistance of the mutant enzymes, it is necessary to measure the kinetics in a precise and highly repeatable fashion over a range of different conditions. In our laboratory, those measurements have been partially automated by the interfacing of a PDP MINC-11 mini-

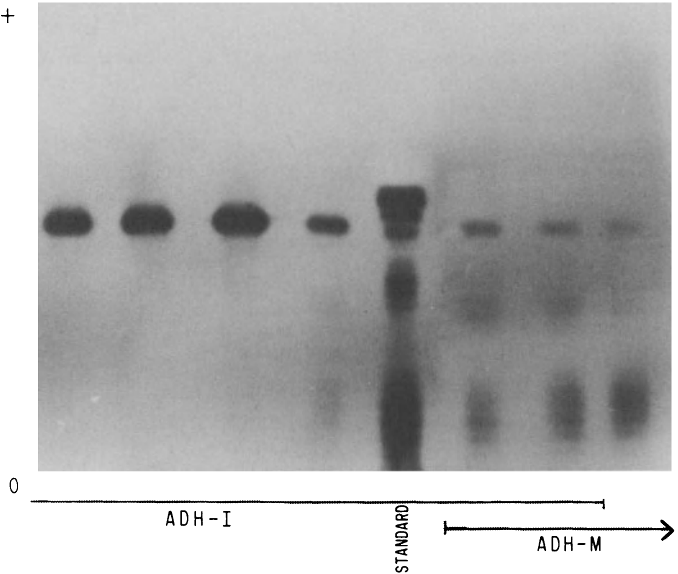

Figure 5. Activity gel showing partial separation of ADH-I and ADH-M. Note that all five hybrid banks of the mitochondrial enzyme elute together on the gradient indicating that the ADH-M "isozymes" are not greatly different from each other in their binding properties.

computer with a Gilford UV-visible spectrophotometer. The mini-computer interfaces directly with a series of programmable modules which simplify both the instrument interfacing and the writing of programs for subsequent data manipulation. A simple pressure switch mounted so that it is closed when the light cover on the spectro-photometer is closed will result in a positive voltage change activating a Schmitt trigger device on the computer's programmable clock. A sweep of analogue data from the spectrophotometer is converted to a large number of digital values and a least squares line is fitted to the data. The change in optical density at 340 nm as the result of the conversion of NAD to NADH or _vice versa_ can thus be followed with precision starting no more than one or two sections after the addition of enzyme to the cuvette. A two second sweep is normally enough to provide an accurate measure of OD changes of as little as 0.01 OD/min. The plot of 1/V against 1/C is presented on the terminal screen at the same time as the computer sends an instruction through a digital-to-analogue converter to change the cuvette in the spectrophotometer in readiness for the next sample. Opening the cover on the spectophotometer has no effect, since a negative voltage change is not registered by the Schmitt trigger. An interactive program has been written which allows the experimenter to drop bad points and to add more replicates at a particular concentration as the experiment proceeds.

Kinetics for each mutant enzyme are normally measured in both reaction directions at pH 8.8 over a range of six to eight substrate and cofactor concentrations. In spite of the fact that highly purified substrate and cofactor are used (it is necessary to column-purify the NADH and distill the acetaldehyde just before use), consistent kinetics in the acetaldehyde-ethanol direction are difficult to obtain. This is particularly the case with mutant enzymes with large K_m, in which high concentrations of NADH and acetaldehyde must be used.

The kinetics obtained can, as an internal check on their consistency, be fitted to Alberty's (1) modification of the Haldane (13) equation:

$$K'_{eq} = \frac{V_f K_{AB_b}}{V_b K_{AB_f}},$$

where f and b refer to the forward (ethanol-acetaldehyde) and back (acetaldehyde-ethanol) reactions respectively, and K_{AB} is a constant (derived from Florini-Vestling plots)(10) which represents the product of the K_m of one substrate and the dissociation constant of the other.

NADH/NAD ratio determination

Determination of this ratio in normally metabolizing cells is difficult, and requires rapid extraction of NAD and NADH without excessive manipulation of the cell culture. We have adapted some methods developed by Brody (unpublished) for extracting these metabolites in hot acid or alkaline ethanol (the former rapidly and differentially destroys NADH and the latter differentially destroys NAD. This is followed by use of a cycling assay (17) to increase the levels of enzymatic products of these cofactors to the point where the products themselves can be assayed. The cycling assay involves malate dehydrogenase and alcohol dehydrogenase with an excess of oxaloacetate and ethanol. If small quantities of NAD are initially present, oxaloacetate is converted to malate by the malate dehydrogenase, and the NADH which results is reconverted to NAD by the alcohol dehydrogenase. A small amount of NADH can also initiate the cycle. If the amount of NAD or NADH present is small, the increase in malate will be linear for a substantial period of time.

The assay is halted by boiling, which destroys both the enzymes and the oxaloacetate, and the malate is then assayed by the NADP-dependent malic enzyme. Small refinements of this assay continue to be made in our laboratory, leading to more accurate measures of the total amounts of NADH and MAD in the cells. The technique is, however, difficult to master and time-consuming, so much effort

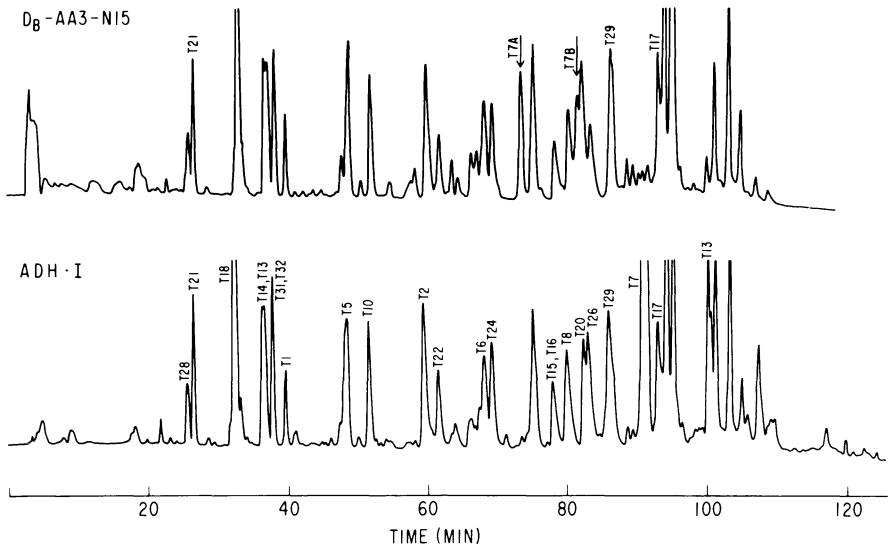

Figure 6. High performance liquid chromatograph (HPLC) separation of peptides in a tryptic digest of a wild type (top) and mutant (bottom) ADH-I. The aqueous phase was 0.1% TFA and the nonaqueous phase was acetonitrile. Separation was carried out on a Waters Bondapak C18 column (10µ particle size), using two Beckman 110A pumps. Effluent was monitored at 220 nm. The gradient was made up of a series of linear gradients: 0-18% CH_3CN over 48 minutes, 18-25% over 30 minutes, 25-40% over 30 minutes, and 40-62% over 20 minutes. The identified tryptic peptides are labeled in the wild type separation. Arrows mark the two new peptides in the mutant which resulted from the cleavage of T7.

is currently being directed towards developing an HPLC assay for NAD and NADH which can be used successfully on whole cell extracts.

Identification of mutant residues

The first two altered residues in ADH-I mutants were determined by conventional methods of tryptic digestion followed by high voltage paper electrophoresis and paper chromatography in two dimensions. We have more recently begun separating peptides by high performance liquid chromatography, which gives remarkable resolution of both tryptic and chymotryptic digests. A number of separation methods using a reverse-phase C-18 column in an Altex two-pump analytical system have been employed. The best resolution, leading to separation of 24 of the 32 tryptic peptides, is obtained with a gradient of 0.1% trifluoracetic acid as the polar solvent and acetonitrile as the nonpolar solvent with the results shown in Figure 6. This

TABLE I

Ratios of NAD to NADH in wild type and mutant yeast strains grown on three different media. Addition of ethanol would be expected to shift the ratio in favor of NADH, while addition of acetaldehyde would be expected to shift it in favor of NAD. The levels of allyl alcohol (in mM) to which some of the strains are resistant are given in brackets. From (34,36).

Strain	Enzyme	Medium: Complete	Complete + 2% EtOH	Complete + 0.04% acetaldehyde
XW517-2D	ADH-I (wild type)	1.09 (0.5)	0.66 (2)	1.49 (0.5)
R234.1-9A	ADH-II (wild type)	1.01 (1)	0.25 (10)	1.51 (0.5)
S288C	ADHI & II (both wild type)	1.21 (0.5)	1.39 (0.5)	1.66 (0.5)
CII-40-80E	ADH-I (mutant)	0.67 (4)	0.27 (20)	0.98 (2)
S-AA-1	ADH-I (mutant)	1.00	0.53	1.82
S-AA-5	ADH-I (mutant)	0.61	0.47	2.37
C-40	ADH-I (mutant)	0.69	0.70	1.24

separation method is particularly advantageous because it results in high yields of the larger peptides. Samples taken directly from the column are frozen at -70° and lyophilized, then taken directly to the amino acid analyzer. Determination of residues involved in charge changes is fairly straightforward using tryptic digests in this system, but determination of alterations which do not lead to electrophoretic mobility changes is more difficult. It may be necessary in some cases to clone the mutant genes and sequence them completely in order to determine the mutational alterations.

RESULTS

Effect of mutant and wild type enzymes on the NAD-NADH ratio

Table I shows the effect of growth on normal medium, and medium containing ethanol or acetaldehyde, on the NAD-NADH ratio of wild type and mutant cells (34,36). Several things are apparent from the table.

First, the ratio changes drastically with addition of metabolites

if only one ADH is present. The shift in the ratio is in the direction expected: addition of ethanol drives the ADH reaction in the direction of acetaldehyde and NADH and enhances the resistance of mutant cells. Addition of acetaldehyde drives the reaction in the opposite direction and reduces the resistance of mutant cells. This reduction of resistance is even apparent when the mutant strains are grown in capped tubes, which allow acetaldehyde to accumulate. The wild type petite, which has ADH-I activity, shows shifts in the ratio of similar magnitude, but the ratio in wild type cells grown on normal medium is not as skewed toward NADH as it is in most of the mutants.

Second, in a wild type strain in which only ADH-II is made, both the resistance to allyl alcohol and the NAD-NADH ratio are increased greatly by growth on ethanol, to levels comparable to some of the mutant strains. The most likely explanation for this lies in the low K_m for ethanol of this isozyme, which is only one tenth that of ADH-I (28). If levels of ethanol inside the cell are low, ADH-II will produce acetaldehyde and NADH at a faster rate than will ADH-I.

Third, in a wild type grande (respiration competent) cell with both isozymes present, strain S288C in the table, the NAD-NADH ratio is stabilized at around 1.2 to 1.6 regardless of the medium on which the cells are grown. This strongly suggests that the presence of two functioning isozymes leads to this stabilization, and suggests in turn that the kinetic properties of the isozymes are such as to cancel out the effect of varying environment when both are present. Preliminary evidence has been presented (28) that there is a small amount of interaction between the substrate and cofactor binding sites of ADH, and that this interaction is opposite in sign in the two isozymes. An elucidation of the mechanism of this stabilization awaits more detailed kinetic studies of the two isozymes, which are currently in progress. It is worth noting in passing that the stabilization of the NAD-NADH ratio is an indication of the importance of ADH to the economy of the cell, and suggests that the enzyme may have functions other than the obvious ones of the oxidation of ethanol and the reduction of acetaldehyde.

The effect of functional point mutations on kinetics

In Table 2, I have listed a series of kinetic parameters obtained from wild type and mutant enzymes. In the mutants marked with an asterisk, the point mutational change is known; in some of the others, it has been approximately localized. The pattern of the data indicates that there is a good deal of independence of the substrate and cofactor binding sites, in that K_m for substrate or for cofactor can be altered quite independently in individual mutants. Further, all the mutants so far localized, though they are distributed throughout the molecule, would be expected to have

TABLE II

Kinetic constants for wild type and mutant enzymes (in moles). The amino acid substitutions for the mutants marked with an asterisk are known or have been approximately localized. Underlined K values are significantly altered from the wild type ADH-I values.

Amino acid substitution	Enzyme	Percent mobility	V_f/V_b	E_{EtOH}	K_{NAD}	K_{acet}	K_{NADH}
	ADH-I	100	0.14	2.4×10^{-2}	2.4×10^{-4}	3.4×10^{-3}	1.4×10^{-4}
	ADH-II	110	0.21	2.7×10^{-3}	1.4×10^{4}	4.5×10^{-5}	2.8×10^{-5}
Trp82 → ?	*S-AA-1	84	0.35	$\underline{4.2 \times 10^{-2}}$	3.1×10^{-4}	$\underline{1.1 \times 10^{-3}}$	1.2×10^{-4}
His44 → Arg	*S-AA-5	93	0.12	2.4×10^{-2}	$\underline{6.0 \times 10^{-5}}$	2.2×10^{-3}	$\underline{6.0 \times 10^{-5}}$
Pro316 → Arg	*C-40	89	0.11	$\underline{1.1 \times 10^{-1}}$	$\underline{2.9 \times 10^{-4}}$	$\underline{8.0 \times 10^{-3}}$	2.0×10^{-4}
	P5-S5-19-7	105	0.10	$\underline{2.2 \times 10^{-1}}$	$\underline{9.5 \times 10^{-4}}$	$\underline{2.9 \times 10^{-2}}$	$\underline{1.1 \times 10^{-3}}$
Trp54 → Arg	*D_B-AA3-N15	95	0.29	$\underline{3 \times 10^{-1}}$	$\underline{4.1 \times 10^{-3}}$	5.3×10^{-3}	$\underline{6.7 \times 10^{-3}}$
	D_A-AA3-N10	94	0.09	$\underline{5.8 \times 10^{-2}}$	$\underline{2.2 \times 10^{-3}}$	$\underline{7.1 \times 10^{-3}}$	$\underline{2.3 \times 10^{-3}}$
	D_B-AA5-E1	100	0.22	3.7×10^{-2}	$\underline{8.6 \times 10^{-4}}$	4.3×10^{-3}	$\underline{9.1 \times 10^{-4}}$

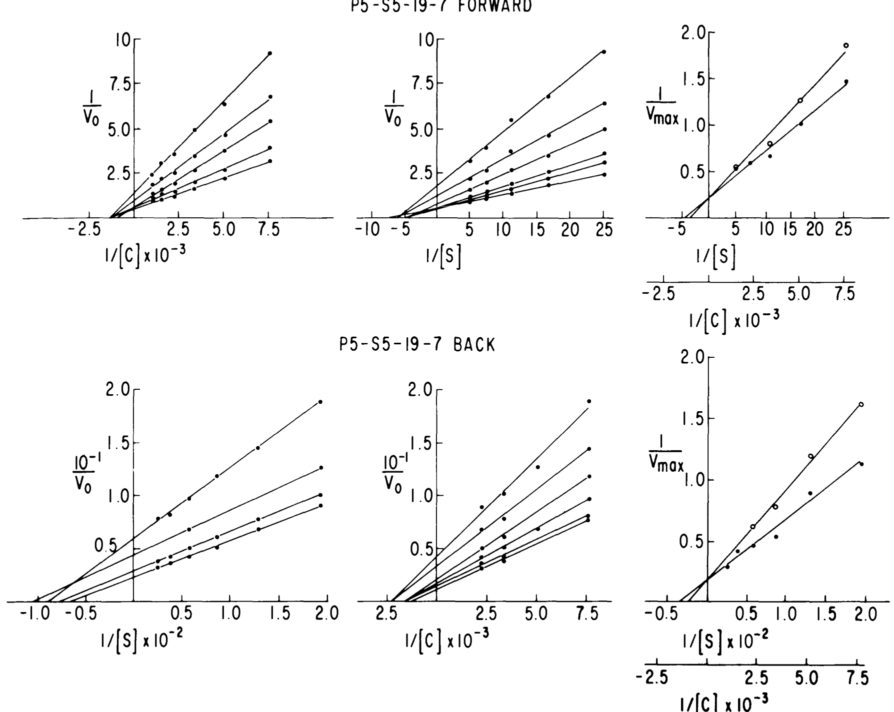

Figure 7. Forward and back kinetics, and secondary plots, for mutant P5-S5-19-7. The open circles in the secondary plot represent the plot of $1/V_{max}$ for substrate against 1/[C] the closed circles represent the plot of $1/V_{max}$ for cofactor against 1/[S]

the effects that they do on the kinetics based on the three-dimensional structure of horse liver ADH.

In Figures 7 and 8, the kinetics of two typical ADH mutants are shown, to illustrate how the kinetic constants are derived. Both mutants show marked alterations in the substrate K_m compared with wild type.

Determination of altered residues

Figure 6 shows a typical separation of tryptic peptides by HPLC, in this case for ADH-I and for the mutant N15. The separation was carried out on a gradient of 0.1% trifluoracetic acid-acetonitrile, which gives the greatest resolution of peptide peaks of the methods tried. Most of the tryptic peptides separable by this technique have been identified by amino acid analysis, and they have been labelled in the ADH-I separation. T1 is the amino terminal tryptic peptide, and T32 is the carboxy terminal peptide.

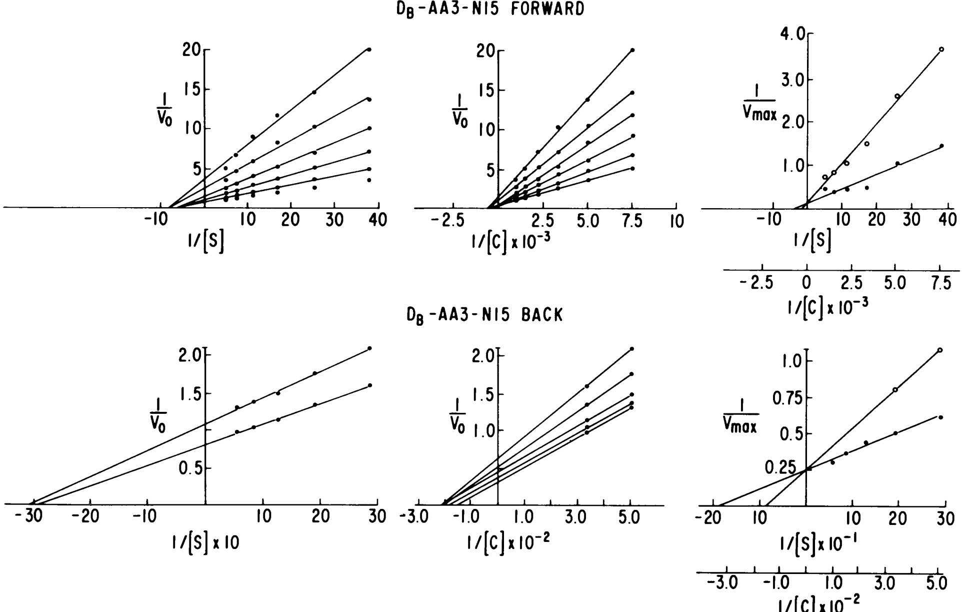

Figure 8. Kinetic data for mutant DB-AA3-N15, presented as in Figure 7.

The large peak of T7 in the ADH-I preparation has been clearly split into two peaks in the mutant (marked with arrows). The split peptides have been examined by amino acid analysis and N-terminal dansylation followed by thin layer chromatography to determine the overall composition and the N-terminal residue. The amino acid compositions of the peptides are given in Table 3. It is apparent that the tryptophan at position 54 has been altered to an arginine in the mutant.

The structural effects of ADH mutants

Because large enough crystals of yeast ADH for X-ray diffraction analysis have yet to be produced, it is necessary to try to understand the effects of mutant substitutions by examining their probable positions estimated from the related residues of horse liver ADH, the structure of which is known to 2.4 Å resolution (11). I have therefore shown, in Figure 9, the positions of the horse liver ADH residues homologous to the altered yeast ADH residues on a diagram provided courtesy of C.-I. Brandén. The three known substitutions are shown, along with the probably positions of two more that have been approximately localized. All the mutants so far determined are in the catalytic domain, but they are found at widely separated sites within that domain.

TABLE III
Amino acid compositions of the tryptic peptides T7A and T7B in mutant D_B-AA3-N15.

	T7A	T7B
N-terminal residue*	tyr	pro
yield (%)	60	40
Composition		
Cys (Cm)	0.76 (1)	
Asx	2.23 (2)	
Thr	-	1.27 (1)
Ser	1.20 (1)	
Pro	-	1.90 (2)
Gly	2.53 (2)	
Ala	1.23 (1)	
Val	1.01 (1)	
Ile	-	
Leu	1.60 (1)	0.93 (1)
Tyr	1.21 (1)	
Lys	-	1.41 (1)
His	2.91 (3)	
Arg	1.04 (1)	
Total	14	5

*Determined by thin layer chromatography of dansylated peptides.

The effect on kinetics of the mutant enzymes varies with the position and type of substitution. Only mutant S-AA-5 shows a reduction in K_m, as a result of the substitution of an arginine for a histidine at position 44. This position corresponds to the arginine at position 47 of the horse liver enzyme, and this arginine has in turn been shown to be important in binding to the pyrophosphate moiety of NAD. The increased strength of binding in mutant S-AA-5 is reflected both in the altered cofactor K_m and the reduced turnover number of this enzyme. Interestingly, this substitution has no effect on substrate binding.

Mutants C-40 and S-AA-1 both have their chief effect on substrate binding. Both are at some distance from the pocket containing the active site zinc atom, and may therefore influence the substrate binding through a propagation of effects. Alternatively, the yeast and horse liver structures may not be congruent at these points, so that the altered yeast residues are actually closer to the binding site. Mutant N-15 has a strong effect on both substrate and cofactor binding, and this is not surprising because the residue altered, trp-54, corresponds to one of the hydrophobic residues lining the active site pocket in the horse liver enzyme, val-57. The effect

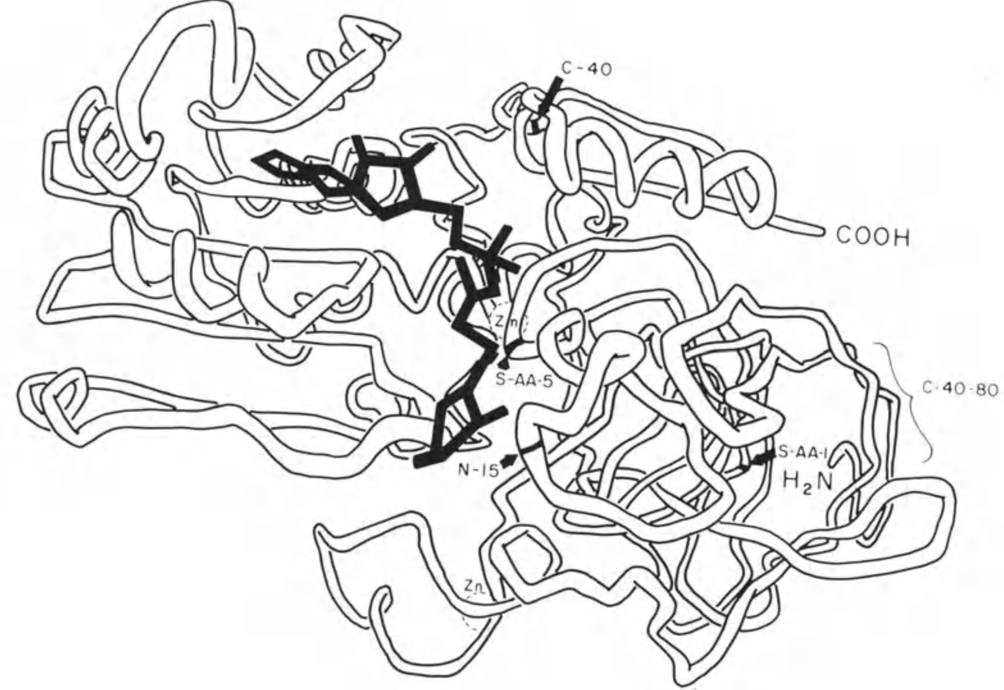

Figure 9. The known or inferred positions of functional yeast ADH mutants, mapped to the positions of the corresponding residues in horse liver ADH. In the diagram, the cofactor-binding domain is to the left and the substrate-binding domain is to the right. The bound cofactor molecule is shown in black. Modified from a diagram kindly provided by C.-I. Brandén.

on the K_m for ethanol is pronounced, increasing it tenfold, but the effect on the K_m for acetaldehyde is much less. This may indicate that the hydrophobic nature of the active site pocket has a greater influence on the passage of ethanol than on that of acetaldehyde.

DISCUSSION

The results so far show that many amino acids in this molecule can be altered to give rise to the small kinetic changes observed. So far, we have examined only mutants in which substitutions resulting in a charge change have occurred, and the only mutants so far determined have resulted in an increase in the negative charge of the molecule. Yet, as has been shown genetically, many other alterations involving uncharged amino acids appear to have occurred. An investigation of one of these mutants, D_B-AA5-E1, by HPLC analysis of both tryptic and chymotryptic digests, has so far failed to reveal

altered peptides, but this mutant does have altered cofactor kinetics compared with the wild type (Table 2). Determining the residues involved in these non-mobility mutations will be a matter of luck, short of cloning and sequencing the mutant genes, but such a large number of them is available that we feel hopeful of solving some of them in the near future.

The most common selective response to allyl alcohol selection is for the mutant enzymes to have raised K_m's for substrate, cofactor or both. In one case, mutant S-AA-5, the K_m for cofactor has been lowered. In no case has the V_{max} in either direction been increased over the wild type. Most of the selective responses, therefore, have resulted in slowing down the rate of conversion of acetaldehyde and NADH to ethanol and NAD at physiological levels of acetaldehyde. This will result in a higher concentration of acetaldehyde in the normally metabolizing cell, and a shift in the NAD-NADH ratio in the direction of NADH.

However, one of the obvious goals of a research project investigating mutant enzyme of alcohol dehydrogenase is to produce strains in which the rate of production of ethanol is enhanced rather than retarded. It is essential for this goal to find selective pressures that will result in a shift of the reaction rates in the cell in the direction of ethanol, and to accomplish this it may be necessary to find specific selective techniques for producing functional mutants in other steps of the pathway such as aldehyde oxidase.

We have made an effort to produce functional mutants of yeast ADH that are conditional -- expressed at high or low temperature or at high osmotic pressure. A few such mutants have been isolated but have yet to be fully characterized genetically. None of these mutants affects the mobility of the ADH. We expect that if some of these can be shown to be localized in the structural gene, they will affect a different subset of residues from that affected by the unconditional mutants.

Attempts to isolate mutants of ADH-II which also confer resistance to allyl alcohol have been made using an $ADR2^C$ mutant isolated by Ciriacy (7). These have not been successful, in part because this strain is already resistant to a high level of allyl alcohol since ADH-II tends to convert ethanol rapidly to acetaldehyde with the concomitant production of NADH.

Perhaps the greatest difficulty lying in the path of the full exploitation of this system is the lack of knowledge of the tertiary structure of this molecule. It would appear, both from our studies and those of Jornvall (15), that the tertiary structures of yeast and horse liver ADH are remarkably similar. Yet, there must be large differences connected with the fact that the functional yeast enzyme is a tetramer while that from liver is a dimer, and other

differences which can explain the very much faster rate of catalysis of the yeast enzyme and the different range of substrate specificities exhibited by the two molecules. On a more subtle level, the parts of the yeast isozymes involved in stabilization of the NAD-NADH ratio may be quite different from those of the liver enzyme. Unfortunately, repeated attempts made in collaboration with the laboratory of C.-I. Brandèn (Zeppezauer, unpublished), using ADH-I freshly purified in our laboratory, have failed to produce large enough crystal for X-ray analysis, though microcrystals have been seen at high pH.

A determination of the tertiary structure of this enzyme, together with the elucidation of the many available functional mutants, should eventually result in an understanding of the basic mechanisms of action of this enzyme which will be equivalent to our knowledge of the hemoglobin molecule. We fully expect that, in addition to its role in NAD-NADH stabilization, other functions of this enzyme will become apparent as the analysis proceeds. There are two points of difference between this system and the hemoglobin system so thoroughly investigated by Perutz and his collaborators (22). The first is that ADH is a catalytic molecule, with the advantage that the mechanism of catalysis can be investigated using mutant substitutions. The second is that, unlike the investigators of the hemoglobin molecule, we are not limited to mutations that have occurred spontaneously in the population. The number of mutants of yeast ADH that can be investigated is limited only by the time and resources of the investigator.

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DISCUSSION

O. RIBBONS: I found your approach very refreshing. While studying the minute changes in protein structure and assessing the effects on substrate binding. Is anything known about changes in thermal stability?

A. WILLS: We find that all the mutants we have looked at are less thermostable than the wild type. There are many other properties we would like to investigate - for example, the effect of inhibitors.

Q. JOHNSON: Is there cross reactivity between the enzyme and the yeast at the cofactor site?

A. WILLS: There is only 25% identity between the two, but compensatory changes indicate that the tertiary structures are very similar.

Q. JOHNSON: Does it make any difference what substrates are utilized by the enzyme?

A. WILLS: The changes in the mutant kinetics are seen with allyl alcohol as well, but we haven't checked other substrates.

Q. JOHNSON: Are the membranes affected in the mutants which show different sensitivity to ethanol?

A. WILLS: Luckily, we have found no membrane mutants! The allyl alcohol selection is remarkably specific.

TOWARDS THE DEVELOPMENT OF MARKETABLE PRODUCTS

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The papers that make up this section deal primarily with fermentative pathways familiar to every microbiologist. Yet the individual papers present their subjects from very different points of view. The perspective of basic research, study for the sake of advancing understanding, is certainly well represented in all of the papers. But, to varying degrees, these papers touch on potential applications of the metabolisms being considered. Implicit in Young's description of alcohol dehydrogenase gene cloning for instance, is the opportunity to manipulate these genes to achieve new approaches to alcohol production. Similarly, Clark's study of _E. coli_ mutants might serve as a model pointing to industrial applications through the profound analysis of the regulation of fermentative metabolism. The other two contributors, in contrast, explicitly discuss potential practical uses of their organisms and related mutants. Hillman describes the occurrence of lactate dehydrogenase deficient _Streptococcus mutans_ and its potential to colonize the mammalian oral cavity with a consequent decrease in dental caries. Higgins reviews a broad range of cooxidative applications of methanotrophs and their enzymes, including some quite novel attempts to supply reducing power to isolated mixed function oxygenases through electrodes and oxidation-reduction dyes.

It is important in a volume discussing the opportunities for microbial production of chemicals through genetic intervention to consider at least cursorily the potential for application of the basic research advances described. A brief consideration of an historical example will illustrate the kinds of considerations that might favor or prevent the realization of an industrial application.

From before World War I until slightly after World War II, a

large percentage of the butanol produced in North America came from the clostridial fermentation of molasses or corn starch (1). This industry could produce net conversion of up to one third of these carbohydrate substrates into marketable solvents, although these were produced as dilute beers containing from 1.7 to 2.4% solvent that had to be concentrated and fractionated by distillation. The fermentation was subject to attack by bacteriophage or by contamination with lactic acid bacteria. The industry eventually fell victim to shifts in the prices of sugar feed stocks and the availability of far cheaper petroleum-based solvent substitutes.

The acetone-butanol industry was the first aseptic industrial fermentation; it also stands as one of the first industrial fermentations to attempt genetic intervention to improve the process, although these interventions were empirical in design. The factor limiting the final concentration of solvent in the beer produced was the tolerance of the organisms for the solvent. Attempts were made to screen for spontaneous and induced mutants that could tolerate higher than normal solvent concentrations. Similarly, it was found that the producer organisms could be "immunized against phage by serial transfer through media containing the virus" (1). This must surely be one of the first examples of the deliberate selection of a relevant mutation to improve an industrial process.

The history of the acetone-butanol fermentation industry teaches that the realization of improvements in industrial fermentation might actually come from genetic manipulation affecting metabolisms far removed from the actual production of the chemical product, for example manipulations affecting the organisms' ability to produce and withstand high concentrations of the desired product or to withstand phage or other competing organisms. Secondly, it teaches that any potential industrial fermentation will have to satisfy overall economic criteria including the relative market value of starting materials and final products, the costs of energy to support the overall process and the availability of competing substitute materials produced by other means. In general, chemical products of microbial origin often fail to meet these criteria because they are produced as dilute solutions and require costly, energy intensive processes to concentrate the product before marketing it. The obvious exceptions are those chemicals that have a high market value that can offset these energy costs, or chemicals that can be produced in no other way except fermentation.

The new era of genetic intervention to create opportunities for chemical production will presumably differ from the past by its theoretical rather than empirical basis. The lessons of past fermentation industries can now, in principle, be re-evaluated from the perspective of a detailed knowledge of the detailed biochemical and genetic basis of the industrially important microbial functions. For instance, genes may be arranged to create organismic tolerance

to high concentrations of product, or the capacity for production may be transplanted to a new microbe with an inherent tolerance. And the mechanistic perspective that has been acquired now opens up the exciting possibility of creating new forms of organisms that will provide us with virtually any microbial metabolite, if we are willing to spend sufficient time and money to characterize the basic biology and to solve the practical problems of bringing the product to the market.

REFERENCE

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THE ALCOHOL DEHYDROGENASE GENES OF THE YEAST, *SACCHAROMYCES CEREVISIAE*: ISOLATION, STRUCTURE, AND REGULATION

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INTRODUCTION

Alcohol dehydrogenase (E.C. 1.1.1.1. ADH) catalyzes the interconversion of an alcohol and an aldehyde with NAD^+ as a cofactor. In most higher organisms that have been studied, several different isozymes of ADH are present. The main function of these isozymes is presumed to be catabolic, to degrade various alcohols or sterols. This presumption is based primarily on the substrate preferences of the various isozymes and the absence of a fermentative pathway for alcohol production during glycolysis. In many organisms there is a distinctive tissue specificity in the distribution of different ADH isozymes.

Unlike the role of ADH in higher organisms, the function of the classical, fermentative isozyme, ADH I, in the baker's yeast *Saccharomyces cerevisiae*, is to oxidize NADH to NAD^+ to allow anaerobic growth. ADH I is coded by the *ADC1* locus (alcohol dehydrogenase constitutive). In addition to ADH I, there are two other ADH isozymes in yeast. ADH II is a glucose-repressed isozyme whose major function is presumed to be ethanol utilization. The structural gene for ADH II is *ADR2* (alcohol dehydrogenase repressible). The third isozyme, mADH, is associated with the mitochondria and is probably encoded by the *ADM* (alcohol dehydrogenase mitochondrial) locus, a nuclear gene.

Through an ingenious series of selections, Ciriacy (4,5,6) was able to isolate mutant yeast strains that contained defective ADH

structural genes and altered regulatory loci. ADH^- mutants can be selected by resistance to allyl alcohol. ADH I^+ revertants and ADH II constitutive mutants can be selected by resistance to antimycin A, a respiratory inhibitor, since only cells having ADH can survive without respiration (23). The ability to select cells lacking or possessing ADH activity is immensely useful in the isolation of ADH mutants and in the cloning of ADH-related genes.

Yeast strains lacking one, two, or all three ADH isozymes were isolated by serial selection on increasing concentrations of allyl alcohol in the medium. All ADH I defective mutants were allelic and mapped at the structural locus ADC1 (4). Mutants with an altered structural gene for ADH II and with altered regulatory loci affecting ADH II synthesis were identified (5,6). Some regulatory alterations were specific for ADH II; others affected several catabolite repressed enzymes including ADH II. Several regulatory loci that decreased ADH II activity contained recessive mutations that were not linked genetically to either ADR2 (the structural gene for ADH II) or to each other. Other mutants had a dominant phenotype and expressed ADH II even in the presence of glucose. Some of these mutations were unlinked to ADR2 ($ADR1^c$) whereas others were tightly linked to ADR2 and were *cis* dominant ($ADR3^c$).

From the above genetic analysis, a positive model of regulation of ADH II synthesis can be proposed in which the product of one of the unlinked regulatory genes, ADR1, interacts with the regulatory locus, ADR3, to turn on transcription of the structural gene ADR2. In order to test this specific model and to characterize this family of yeast isozymes more fully both in terms of regulation and structure, we have isolated and begun to characterize the genes involved in ADH synthesis.

RESULTS

Cloning

1. ADC1. The first yeast ADH gene to be cloned was ADC1 (21), the structural gene for the fermentative isozyme ADH I. The gene was cloned by transformation of a yeast strain totally lacking ADH activity (ADH-null) with a plasmid pool containing yeast DNA fragments and selection for transformants able to grow in the presence of antimycin A. The yeast vector used was YRp7 which contains the *E. coli* vector pBR322, the yeast TRP1 gene, and a yeast origin of replication. Selection in *E. coli* can be based on either tet^R or amp^R and transformed yeast can be selected for growth in the absence of tryptophan (19).

2. ADR2. The structural gene for ADH II, ADR2 was cloned in two different ways. In the first way we used yeast transformation

and complementation of function, as described above. However, the recipient yeast strain for transformation had to be glucose-insensitive for ADH II expression since spheroplast regeneration is very poor in the absence of glucose. Expression of ADH II in the spheroplasts was achieved by using a strain containing a mutation in the regulatory locus, ADR1, which allows constitutive ADHII expression. The recipient strain contained a defective structural gene for ADR2 as well as defective ADH I and mADH genes. The second technique that was used for cloning ADR2 was based on the ability of the ADC1 and ADR2 genes to crosshybridize. This was predicted on the basis of the amino acid sequence homology between ADH I and ADH II (24). The second technique has the advantage that a total genomic pool of yeast DNA fragments in a yeast vector does not have to be constructed. Instead, a restriction enzyme fragment was identified by Southern transfer hybridization, cut out, cloned in E. coli, and identified by colony hybridization. Utilizing these two techniques a series of plasmids was isolated that contained the wild-type ADR2 locus (22).

3. ADR3. Utilizing crosshybridization we have also isolated the ADR2 locus linked to several cis-acting constitutive regulatory mutations, ADR3^c. The isolation of one of these mutant DNAs has already been described (22). As reported in more detail below, these mutations are most commonly due to insertion of a transposable yeast element Ty (3,22) adjacent to the 5' end of ADR2. By cloning four more of these mutant DNAs we have established the generality of the phenomenon and have determined the precise site and orientation of these insertions.

4. ADR1. Cloning by transformation of yeast and complementation of function allows one to isolate genes whose functions are unknown or for which there is no product that can be detected (18). We have cloned the regulatory gene ADR1 by this technique. This gene is a positive regulator of ADR2 (5,6). We first cloned the mutant regulatory gene ADR1-5^c which allows selection for ADH II in the presence of glucose. A library of yeast DNA fragments from a strain of the genotype adc1-11 ADR2 adm ADR1-5^c was constructed in the vector YRp7. This plasmid pool was used to transform a strain that had the genotype adc1-11 ADR2 adm trp1 adr1-1 and which did not synthesize ADH II. Transformants were isolated which expressed ADH II constitutively and thus putatively contained plasmid with the ADR1-5^c gene.

This plasmid was shown to contain the ADR1-5^c gene by obtaining stable transformants in which the plasmid had presumably integrated into the yeast genome. The site of integration was shown by genetic means to be at the ADR1 locus. Specifically, the stable transformant was mated with a strain of the genotype adc1-11 adm ADR2 trp1 ADR1 and the resultant spores from this cross were dissected. Table I shows that in all 40 tetrads analyzed the ADR1-5^c allele was linked

TABLE I
Genetic Linkage of Integrated Plasmid

Gene pair	Ascus type PD	NPD	T	Calculated Map Distance
ADR1-5^c/adr1-1	39	0	0	≤ 1.25
ADR1-5^c/TRP1	24	0	0	≤ 4.0
ADR1-5^c/pBR322	14	0	0	≤ 6.7

Test crosses were

YRp7-ADR1-5^c-int/adc1-11 ADR2 adr1-1

X

adc1-11 ADR2 ADR1

TABLE II
ADH Activities for Cells with Plasmids Containing ADR1 and ADR1-5^c Genes

Strain	Specific Activity (mu/mg) Medium containing Glucose	Ethanol
a. Transformed 500-16		
500-16/YRp7-ADR1-5^c	200	360
500-16/YRp7-ADR1	30	370
500-16/YRp7-ADR5^c (integrant)	220	3800
500-16/YRp7-ADR1 (integrant)	30	800
b. Parental strains		
R234 (ADR1-5^c)	200	4000
43-2B (ADR1)	40	2000
500-16 (adr1-1)	5	50

Strain 500-16 has the genotype a trp1 his4·ura1 adc1-11 ADR2 adr1-1 adm
Enzyme activities were determined after growing cells overnight in medium containing either 8% glucose or 3% ethanol. All values are the average of at least six determinations.

to the adr1-1 allelle. In 24 of these tetrads which were scored the TRP+ phenotype from the original plasmid also co-segregated with ADR1-5^c. Thus, the site of insertion of the ADR1-5^c containing plasmid was shown to map near the ADR1 locus.

To isolate the wild-type ADR1 gene a library of yeast fragments containing this gene was screened by crosshybridization essentially as described above for isolation of ADR2.

Table II shows that the phenotype of yeast strains with stable integrants and those transformed with these plasmids qualitatively agrees with the expected phenotype of the gene in the parental strains.

Comparison of ADC1 and ADR2 Nucleotide Sequences

1. Coding sequence comparisons. Isolation of the structural genes for ADH I and ADH II, ADC1 and ADR2 respectively, has allowed us to compare the nucleotide sequences of the related isozymes. The amino acid sequence of ADH I and 82 percent of ADH II have been published (15,24). Bennetzen (1) determined the nucleotide sequence of all of the coding region of ADC1, 700bp of 5' flanking region and 300bp of 3' flanking DNA. We have recently determined the nucleotide sequence of the structural region of ADR2, 300bp of 5' flanking DNA and 900bp of 3' flanking DNA. Neither gene has an intervening sequence. The complete nucleotide sequence of the coding regions of ADC1 and ADR2 are shown in Figure 1, together with the amino acid sequence deduced from the nucleotide sequence. The underlined amino acids differ from the published amino acid sequence (24). Some of these differences are probably due to strain differences between the yeast from which the protein sequence was determined and the yeast from which the genes were cloned. The two genes are 95% homologous in amino acid sequence and 90% homologous in nucleotide sequence.

The most interesting feature of the comparison of ADC1 and ADR2 nucleotide sequences in the protein coding region is the identity of the sequences between nucleotide residues 193 and 355. This identity can be explained in various ways. A strong selective pressure could be operating on this region of the gene but one which is not acting solely on the protein product since even the third positions of each codon are conserved. Codon bias (see below) is not an adequate explanation for conservation of the third position in each codon since many of the amino acids in this region are not coded by triplets with a biased codon usage. Alternatively, these regions of ADC1 and ADR2 could have been exchanged in recent times by gene conversion. A similarly conserved region, but smaller in extent, was observed between the two cloned cytochrome genes of yeast, CYC1 and CYC7 (17).

ADC1

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ATG TCT ATC CCA GAA ACT CAA AAA GGT GTT ATC TTC TAC GAA TCC CAC GGT AAA TTG GAA CAC AAG GAT ATT CCA
Met Ser Ile Pro Glu Thr Gln Lys Gly Val Ile Phe Tyr Glu Ser His Gly Lys Leu Glu His Lys Asp Ile Pro

GTT CCA AAG CCA AAG GCC AAC GAA TTG TTG ATC AAC GTT AAG TAC TCT GGT GTC TGT CAC ACC GAC TTG CAC GCT
Val Pro Lys Pro Lys Ala Asn Glu Leu Leu Ile Asn Val Lys Tyr Ser Gly Val Cys His Thr Asp Leu His Ala

TGG CAC GGT GAC TGG CCA TTG CCA GTT AAG CTA CCA TTA GTC GGT GGT CAC GAA GGT GCC GGT GTC GTT GTC GGC
Trp His Gly Asp Trp Pro Leu Pro Val Lys Leu Pro Leu Val Gly Gly His Glu Gly Ala Gly Val Val Val Gly

ATG GGT GAA AAC GTT AAG GGC TGG AAG ATC GGT GAC TAC GCC GGT ATC AAA TGG TTG AAC GGT TCT TGT ATG GCC
Met Gly Glu Asn Val Lys Gly Trp Lys Ile Glv Asp Tyr Ala Gly Ile Lys Trp Leu Asn Gly Ser Cyc Met Ala

TGT GAA TAC TGT GAA TTG GGT AAC GAA TCC AAC TGT CCT CAC GCT GAC TTG TCT GGT TAC ACC CAC GAC GGT TCT
Cys Glu Tyr Cys Glu Leu Gly Asn Glu Ser Asn Cys Pro His Ala Asp Leu Ser Gly Tyr Thr His Asp Gly Ser

TTC CAA CAA TAC GCT ACC GCT GAC GCT GTT CAA GCC GCT CAC ATT CCT CAA GGT ACC GAC TTG GCC CAA GTC GCC
Phe Gln Gln Tyr Ala Thr Ala Asp Ala Val Gln Ala Ala His Ile Pro Gln Gly Thr Asp Leu Ala Gln Val Ala

CCC ATC TTG TGT GCT GGT ATC ACC GTC TAC AAG GCT TTG AAG TCT GCT AAC TTG ATG GCC GGT CAT TGG GTT GCC
Pro Ile Leu Cys Ala Gly Ile Thr Val Tyr Lys Ala Leu Lys Ser Ala Asn Leu Met Ala Gly His Trp Val Ala

ATT TCC GGT GCT GCC GGT GGT CTA GGT TCT TTG GCT GTT CAA TAC GCC AAG GCT ATG GGT TAC AGA GTC TTG GGT
Ile Ser Gly Ala Ala Gly Gly Leu Gly Ser Leu Ala Val Gln Tyr Ala Lys Ala Met Gly Tyr Arg Val Leu Gly

ATT GAC GGT GGT GAA GGT AAG GAA GAA TTA TTC AGA TCC ATC GGT GGT GAA GTC TTC ATT GAC TTC ACT AAG GAA
Ile Asp Gly Gly Glu Gly Lys Glu Glu Leu Phe Arg Ser Ile Gly Gly Glu Val Phe Ile Asp Phe Thr Lys Glu

AAG GAC ATT GTC GGT GCT GTT CTA AAG GCC ACT GAC GGT GGT GCT CAC GGT GTC ATC AAC GTT TCC GTT TCC GAA
Lys Asp Ile Val Gly Ala Val Leu Lys Ala Thr Asp Gly Gly Ala His Gly Val Ile Asn Val Ser Val Ser Glu

GCC GCT ATT GAA GCT TCT ACC AGA TAC GTT AGA GCT AAC GGT ACC ACC GTT TTG GTC GGT ATG CCA GCT GGT GCC
Ala Ala Ile Glu Ala Ser Thr Arg Tyr Val Arg Ala Asn Gly Thr Thr Val Leu Val Gly Met Pro Ala Gly Ala

AAG TGT TGT TCT GAT GTC TTC AAC CAA GTC GTC AAG TCC ATC TCT ATT GTT GGT TCT TAC GTC GGT AAC AGA GCC
Lys Cys Cys Ser Asp Val Phe Asn Gln Val Val Lys Ser Ile Ser Ile Val Gly Ser Tyr Val Gly Asn Arg Ala

GAC ACC AGA GAA GCT TTG GAC TTC TTC GCC AGA GGT TTG GTC AAG TCT CCA ATC AAG GTT GTC GGC TTG TCT ACC
Asp Thr Arg Glu Ala Leu Asp Phe Phe Ala Arg Gly Leu Val Lys Ser Pro Ile Lys Val Val Gly Leu Ser Thr

TTG CCA GAA ATT TAC GAA AAG ATG GAA AAG GGT CAA ATC GTT GGT AGA TAC GTT GTT GAC ACT TCT AAA TAA
Leu Pro Glu Ile Tyr Glu Lys Met Glu Lys Gly Gln Ile Val Gly Arg Tyr Val Val Asp Thr Ser Lys och
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Figure 1. The nucleotide sequences of ADC 1 and the derived amino acid sequence. The underlined amino acids indicate changes from the published amino acid sequence.

Codon usage in the yeast genes coding for the major glycolytic enzymes is decidedly non-random (1,14). It appears that codon preference is correlated with the abundance of isoaccepting tRNA species (1,14). Proteins which constitute a large fraction of the cellular protein utilize preferentially those codons which can be decoded by the most abundant isoacceptor species of tRNA, whereas minor proteins such as iso-2-cytochrome c show no such codon bias (14,17). The codon usage of ADR2 is shown in Table III which includes the codon usage of ADC1 for comparison (1). The codon bias for ADR2 is very similar to that of ADC1, as would be expected since the two genes are very homologous. There is, however, slightly less codon bias in ADR2.

2. Non-coding sequences. A comparison of the 5'-non-coding regions of ADC1 and ADR2 is shown in Figure 2. These sequences

ADR2

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ATG TCT ATT CCA GAA ACT CAA AAA GCC ATT ATC TTC TAC GAA TCC CAC GGC AAG TTG GAG CAT AAG GAT ATC CCA
Met Ser Ile Pro Glu Thr Gln Lys Ala Ile Ile Phe Tyr Glu Ser His Gly Lys Leu Glu His Lys Asp Ile Pro

GTT CCA AAG CCA AAG GCC AAC GAA TTG TTA ATC AAC GTC AAG TAC TCT GGT GTC TGC CAC ACC GAT TTG CAC GCT
Val Pro Lys Pro Lys Ala Asn Glu Leu Leu Ile Asn Val Lys Tyr Ser Gly Val Cys His Thr Asp Leu His Ala

TGG CAT GGT GAC TGG CCA TTG CCA ACT AAG TTA CCA TTA GTT GGT GGT CAC GAA GGT GCC GGT GTC GTT GTC GGC
Trp His Gly Asp Trp Pro Leu Pro Thr Lys Leu Pro Leu Val Gly Gly His Glu Gly Ala Gly Val Val Val Gly

ATG GGT GAA AAC GTT AAG GGC TGG AAG ATC GGT GAC TAC GCC GGT ATC AAA TGG TTG AAC GGT TCT TGT ATG GCC
Met Gly Glu Asn Val Lys Gly Trp Lys Ile Gly Asp Tyr Ala Gly Ile Lys Trp Leu Asn Gly Ser Cys Met Ala

TGT GAA TAC TGT GAA TTG GGT AAC GAA TCC AAC TGT CCT CAC GCT GAC TTG TCA GGT TAC ACC CAC GAC GGT TCT
Cys Glu Tyr Cys Glu Leu Gly Asn Glu Ser Asn Cyc Pro His Ala Asp Leu Ser Gly Tyr Thr His Asp Gly Ser

TTC CAA GAA TAC GCT ACC GCT GAC GCT GTT CAA GCC GCT CAC ATT CCT CAA GGT ACT GAC TTG GCT GAA GTC GCG
Phe Gln Glu Tyr Ala Thr Ala Asp Ala Val Gln Ala Ala His Ile Pro Gln Gly Thr Asp Leu Ala Glu Val Ala

CCA ATC TTG TGT GCT GGT ATC ACC GTA TAC AAG GCT TTG AAG TCT GCC AAC TTG AGA GCA GGC CAC TGG GCG GCC
Pro Ile Leu Cys Ala Gly Ile Thr Val Tyr Lys Ala Leu Lys Ser Ala Asn Leu Arg Ala Gly His Trp Ala Ala

ATT TCT GGT GCT GCT GGT GGT CTA GGT TCT TTG GCT GTT CAA TAT GCT AAG GCG ATG GGT TAC AGA GTC TTA GGT
Ile Ser Gly Ala Ala Gly Gly Leu Gly Ser Leu Ala Val Gln Tyr Ala Lys Ala Met Gly Tyr Arg Val Leu Gly

ATT GAT GGT GGT CCA GGA AAG GAA GAA TTG TTT ACC TCG CTC GGT GGT GAA GTA TTC ATC GAC TTC ACC AAA GAG
Ile Asp Gly Gly Pro Gly Lys Glu Glu Leu Phe Thr Ser Leu Gly Gly Glu Val Phe Ile Asp Phe Thr Lys Glu

AAG GAC ATT GTT AGC GCA GTC GTT AAG GCT ACC AAC GGC GGT GCC CAC GGT ATC ATC AAT GTT TCC GTT TCC GAA
Lys Asp Ile Val Ser Ala Val Val Lys Ala Thr Asn Gly Gly Ala His Gly Ile Ile Asn Val Ser Val Ser Glu

GCC GCT ATC GAA GCT TCT ACC AGA TAC TGT AGG GCG AAC GGT ACT GTT GTC TTG GTT GGT TTG CCA GCC GGT GCA
Ala Ala Ile Glu Ala Ser Thr Arg Tyr Cys Arg Ala Asn Gly Thr Val Val Leu Val Gly Leu Pro Ala Gly Ala

AAG TGC TCC TCT GAT GTC TTC AAC CAC GTT GTC AAG TCT ATC TCC ATT GTC GGC TCT TAC GTG GGG AAC AGA GCT
Lys Cys Ser Ser Asp Val Phe Asn His Val Val Lys Ser Ile Ser Ile Val Gly Ser Tyr Val Gly Asn Arg Ala

GAT ACC AGA GAA GCC TTA GAT TTC TTT GCC AGA GGT CTA GTC AAG TCT CCA ATA AAG GTA GTT GGC TTA TCC AGT
Asp Thr Arg Glu Ala Leu Asp Phe Phe Ala Arg Gly Leu Val Lys Ser Pro Ile Lys Val Val Gly Leu Ser Ser

TTA CCA GAA ATT TAC GAA AAG ATG GAG AAG GGC CAA ATT GCT GGT AGA TAC GTT GTT GAC ACT TCT AAA TAA
Leu Pro Glu Ile Tyr Glu Lys Met Glu Lys Gly Gln Ile Ala Gly Arg Tyr Val Val Asp Thr Ser Lys och
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are aligned to maximize the homology observed in three regions: the 8 nucleotides just outside the structural gene, the nucleotides surrounding the mRNA 5' ends at positions -27 and -37 (ADC1) and -55 and -57 (ADR2), and the sequences around the Goldberg-Hogness "TATAA" sequences at positions -128 and -162 for ADC1 and ADR2, respectively. There is a stretch of nearly complete homology (28/31 nucleotides) surrounding the putative initiation sites for transcription (see below) and 16 out of 18 nucleotides surrounding the TATAA boxes are homologous. There is a conserved region (12/13 nucleotides) in between these regions as well. The distance between the ATG of the structural gene and the position of the 5' end of the ADR2 mRNA is greater than the analogous distance for ADC1 in part because of the direct repetition in ADR2 of the sequence CAACTAT located between positions -11 and -17 in ADC1 This sequence is present once in ADC1 and three times in ADR2. Since there is so much sequence

TABLE III
Codon Selection in ADC 1 and ADR 2

UUU Phe 0	UCU Ser 14	UAU Tyr 0	UGU Cys 8
UUC Phe 8	UCC Ser 7	UAC Tyr 13	UGC Cys 0
UUA Leu 2	UCA Ser 0	UAA och 1	UGA umb 0
UUG Leu 19	UCG Ser 0	UAG amb 0	UGC Trp 5
CUU Leu 0	CCU Pro 2	CAU His 1	CGU Arg 0
CUC Leu 0	CCC Pro 1	CAC His 10	CGC Arg 0
CUA Leu 3	CCA Pro 10	CAA Gln 9	CGA Arg 0
CUG Leu 0	CCG Pro 0	CAG Gln 0	CGG Arg 0
AUU Ile 9	ACU Thr 4	AAU Asn 0	AGU Ser 0
AUC Ile 12	ACC Thr 10	AAC Asn 11	AGC Ser 0
AUA Ile 0	ACA Thr 0	AAA Lys 4	AGA Arg 8
AUG Met 7	ACG Thr 0	AAG Lys 20	AGG Arg 0
GUU Val 19	GCU Ala 19	GAU Asp 2	GGU Gly 41
GUC Val 17	GCC Ala 16	GAC Asp 14	GGC Gly 3
GUA Val 0	GCA Ala 0	GAA Glu 20	GGA Gly 0
GuG Val 0	GCG Ala 0	GAG Glu 0	GGG Gly 0

ADC1

UUU Phe 2	UCU Ser 13	UAU Tyr 1	UGU Cys 6
UUC Phe 6	UCC Ser 7	UAC Tyr 12	UGC Cys 2
UUA Leu 7	UCA Ser 1	UAA och 1	UGA umb 0
UUG Leu 15	UCG Ser 1	UAG amb 0	UGC Trp 5
CUU Leu 0	CCU Pro 2	CAU His 2	CGU Arg 0
CUC Leu 1	CCC Pro 0	CAC His 10	CGC Arg 0
CUA Leu 2	CCA Pro 12	CAA Gln 6	CGA Arg 0
CUG Leu 0	CCG Pro 0	CAG Gln 0	CGG Arg 0
AUU Ile 9	ACU Thr 5	AAU Asn 1	AGU Ser 1
AUC Ile 12	ACC Thr 9	AAC Asn 1	AGC Ser 1
AUA Ile 1	ACA Thr 0	AAA Lys 4	AGA Arg 7
AUG Met 5	ACG Thr 0	AAG Lys 20	AGG Arg 1
GUU Val 16	GCU Ala 18	GAU Asp 6	GGU Gly 32
GUC Val 12	GCC Ala 13	GAC Asp 9	GGC Gly 8
GUA Val 3	GCA Ala 3	GAA Glu 18	GGA Gly 1
GuG Val 1	GCG Ala 4	GAG Glu 3	GGG Gly 1

ADR2

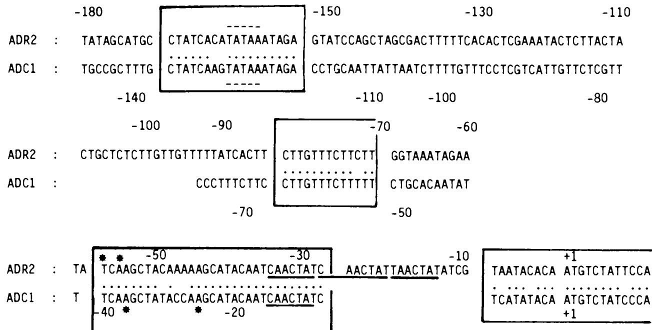

Figure 2. Comparison of nucleotide sequences in the 5' flanking region of ADC 1 and ADR 2. The two sequences have been aligned to obtain maximum homology indicated by the boxed regions. In those areas identical bases are highlighted by dashed lines and the solid underlining indicates a duplicated sequence. The RNA 5' ends are indicated by an asterisk.

homology between positions -1 and -160 for ADC1 and ADR2, it seems likely that this whole region, as well as the structural gene, was included in the duplication event which preceded divergence. The homology as well as the sites of insertion of transposable elements that alter ADR2 regulation suggests that the nucleotide sequences utilized for regulating ADR2 expression are located 5' to the TATAA sequence.

Homology between ADC1 and ADR2 3' to the coding regions extends only to the first triplet of bases beyond the UAA termination codon. Beyond this position there is no extensive nucleotide homology that has been detected.

Transcription Mapping of ADC1 and ADR2

The mRNA 5' ends for ADC1 and ADR2 are indicated by the asterisks in Figure 2. Bennetzen (pers. comm.) and Ammerer (pers. comm.) found two different 5' ends for ADC1 mRNA using S1 nuclease mapping (2). The location of these ends corresponded to positions -27 and -37 of the DNA sequence. The 5' ends of ADR2 were determined similarly. An S1 analysis using a single-stranded fragment of the cloned ADR2 gene is shown in Figure 3. There are two prominent bands that are aligned with DNA postions -55 and -57 on the DNA sequencing gel run adjacent to the S1 mapping lanes. Thus, it appears that both ADC1 and ADR2 have two major transcripts. A third and much larger but less abundant ADC1 transcript is observed in some growth conditions (Ammerer, pers. comm.).

An interesting artifact that is caused by the homology between

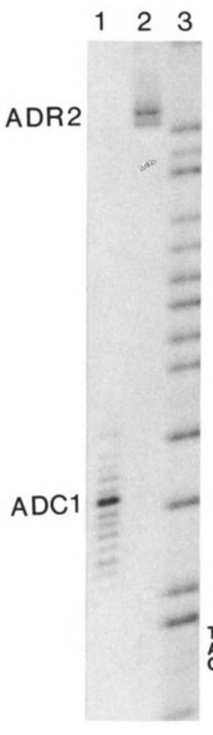

Figure 3. Determination of the 5' end of ADR2 mRNA by SI mapping. The methods of Berk and Sharp (2) as modified by Nasmyth et. al. (18) was used to determine the 5' end of the ADR2 transcript. A single stranded DNA fragment was labeled with ^{32}P at its 5' end which is located 40 base pairs within the coding sequence of ADR2. Using the conditions described by Nasmyth et. al. (18), this DNA fragment was hybridized to yeast RNA, treated with nuclease SI, then sodium hydroxide, and analysed on a polyacrylamide DNA sequencing gel. In lane 1, RNA was isolated from cells grown on glucose and in lane 2 RNA was isolated from cells grown on ethanol containing medium. Lane 3 contains fragments after guanine specific cleavage of the DNA fragement described above as described by Maxam and Gilbert (16a). TAC designates the location of the translation start site. ADC1 and ADR2 indicate the positions of bands reflecting ADC1 and ADR2 transcript levels.

ADC1 and ADR2 is shown in Figure 3. The radioactive hybridization probe for the S1 mapping extends 40 nucleotides into the coding sequence of ADR2. Because there is extensive nucleotide homology between ADC1 and ADR2, mRNAs for both genes can hybridize with the probe. However, S1 nuclease cuts the DNA that is hybridized to the ADC1 mRNA at positions of extensive non-homology. This give rise to the lower molecular weight DNA fragments labeled

"ADC1 mRNA" in Figure 3. These fragments of DNA correspond to S1 nuclease cleavage of the probe at positions beginning around nucleotide -9, the position at which ADC1 and ADR2 diverge from homology (Figure 2). This crosshybridization has been used to obtain a qualitative estimate of the relative abundance of ADC1 and ADR2 RNAs isolated from cells grown in glucose-containing medium and in ethanol containing medium (Section "Regulation of Expression of ADC1 and ADR2").

We do not know whether these ends of the mature mRNAs are the transcript initiation sites or processing sites. However, evidence presented in the next section suggests that the minor large ADC1 transcript is not an essential precursor to the two major ADC1 transcripts. The exact end points are not known since S1 does not create perfectly flush ends if the 5' end is capped. We have not detected by northern gel analysis a larger ADR2 transcript that could be a precursor to the transcripts shown in Figure 3.

In Vitro Mutagenesis of ADC1

A series of deletion mutants lacking all or part of the nucleotide sequences flanking the 5' end of ADC1 have been created by digestion with exonuclease III. The plasmids containing the

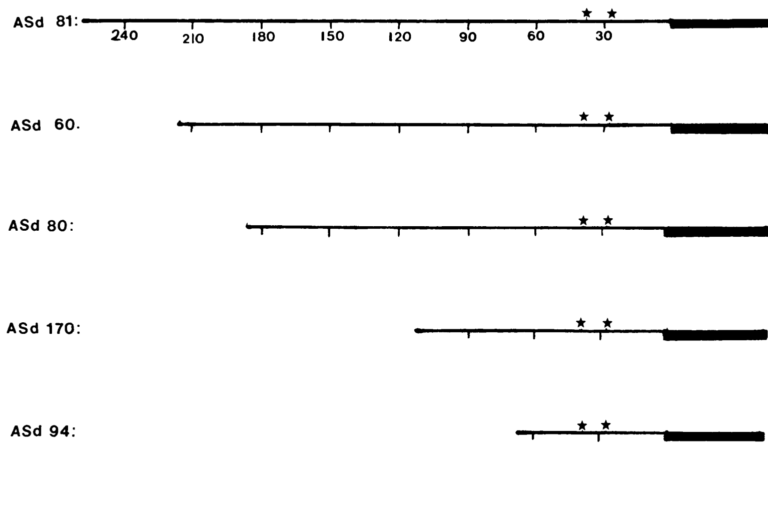

Figure 4. *In vitro* generated deletions in the 5' flanking region of ADC1. Deletions were created by exonuclease III digestion starting at a unique Taq I restriction enzyme site 260 bp from the ATG of ADC1. The nucleotides remaining 5' to the gene are shown by the light black line. The heavy black line represents the ADC1 gene. The asterisks denote the mRNA 5' ends.

TABLE IV
a/α Suppression of Yeast Alcohol
Dehydrogenase Constitutive Mutations

Strain	bp Yeast 5' sequence	AT	AA	ADH activity (mu/mg)	% Trp+	ADH activity (corrected)*
79-72C	wt	+	-	3220	--	--
YRp9T6	-1700	+	-	7360	17	43200
YRpTF5	-260	+	-	3096	32	9625
YRpASd-181	-260	+	-	805	24	3288
YRpASd-160	-220	+	-	1610	45	3542
YRpASd-80	-180	+	-	404.8	38	1024
YRpASd-170	-150	+	-	2898	30	9607
YRpASd-94	-70	+	+	110.4	62	152
YrpASd-118	+20	-	+	16.1	28	--
No plasmid	-	-	+	5	0	5

*correction: $\frac{\text{ADH activity -- 16.1 (background ADH2 activity)}}{\text{\%trp+}} \times 100$

deletions were constructed in such a way that each one contained a common vector sequence abutting the deletion end point. These deletions have end points that range from position -260 to +8 (within the structural gene) (Figure 4). An ADH null strain of yeast was transformed with the parental plasmid and the plasmids containing deleted 5' ends. Table IV shows the ADH activities in the transformants. Because the YRp7 plasmid is unstable, the enzyme activities are also presented as the "corrected" ADH activity, which was obtained by dividing the ADH specific activity by the fraction of cells capable of growth in the absence of tryptophan (i.e., retaining the plasmid). However, some cells may have lost the ability to grow in the absence of tryptophan but still contain ADH activity. Because of this ambiguity the enzyme activities are only of qualitative value.

The enzyme activities show, even with the qualification, that the deletion removing sequences 5' to position -70 reduces enzyme activity at least 10-fold whereas the deletion ending at position -150 has a minor effect on ADH I activity. A TATAA box, which is thought to be involved in transcription initiation, is located at position -128. As expected, the deletion ending within the structural gene abolishes enzyme activity.

These same deletion mutants were recloned into a vector containing the centromere of chromosome III (CEN3; 8). This plasmid

is stably maintained at one copy per cell. Preliminary evidence obtained from transformants containing these plasmids gave data consistent with that shown in Table IV.

Since none of these plasmids contain 5' flanking sequences extending further than position -260, we conclude that the minor large ADC1 mRNA sometimes observed is not a prerequisite for transcription of ADC1.

Regulation of Expression of ADC1 and ADR2

One of the major reasons for choosing to study ADC1 and ADR2 was the distinctive regulation of these two closely related genes. Based on analysis of enzyme activities, it was believed that ADC1 was expressed constitutively and that ADR2 was repressed by glucose. The isolation of cloned DNA sequences of each of these genes was a prerequisite to analyzing their regulation at the transcriptional level. Initially, however, we studied their regulation by measuring mRNA levels by *in vitro* translation (9). In summary, this work indicated that functional mRNA levels from ADR2 were decreased by growth on glucose, and that the ADR1 gene product was essential for the synthesis of derepressed levels of functional ADH II mRNA (9). The initial work was done in a strain lacking a functional ADC1 gene in order to be able to measure ADH II enzyme activities as well as protein and mRNA levels. Subsequent studies in wild-type strains in which both ADC1 and ADR2 were functional has confirmed the results for ADR2. In addition, these studies showed that ADC1 was also regulated but in an inverse fashion to ADR2. As shown in Figure 5, when cells growing on glucose are shifted to medium containing a non-fermentable carbon source (ethanol) ADR2 mRNA is derepressed but ADC1 mRNA is repressed. This analysis has been performed using two different techniques to distinguish between ADHI and ADH II proteins with basically the same results obtained in each case.

Once the ADC1 and ADR2 genes were cloned it was possible to analyze ADC1 and ADR2 mRNA levels by hybridization to the cloned DNAs. Homology between the two genes which extends into the 5' leader sequences complicates the analysis and prevents a straightforward comparison by using radioactive RNA. We have done quantitative S1 analysis by performing the hybridization reaction in DNA excess (2) which should allow both ADC1 and ADR2 mRNA to hybridize to the ADR2 probe to an extent proportional to its concentration in the population of RNA, irrespective of the length of the homologous hybridizing region. This should provide a qualitative estimate of the relative amount of ADC1 and ADR2 RNA in the two different growth conditions we are interested in: glucose (repressing conditions for ADR2) and ethanol or glycerol (derepressing conditions for ADR2). The results of such an S1

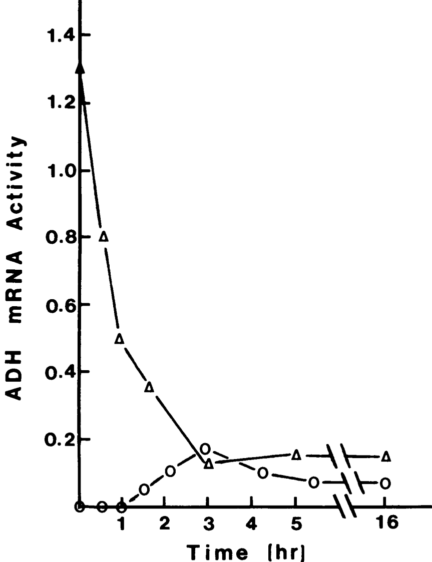

Figure 5. ADH mRNA activity during growth of yeast on ethanol. The values represent the percent ADHI or ADHII protein synthesis of total protein synthesis as directed by yeast RNA in the wheat germ cell-free system. RNA was extracted at the terms indicated after transferring cells grown overnight in 2% glucose to fresh media containing 3% ethanol. ADHI: Δ———Δ; ADHII: O———O.

analysis are shown in Figure 3. It is clear that the relative amounts of ADC1 and ADR2 are reversed in glucose and glycerol. Very little ADR2 transcript is detected in RNA extracted from glucose-grown cells and ADC1 RNA is relatively abundant, and the relative levels are reversed in RNA extracted from glycerol-grown cells. These results confirm the analysis that was done by measuring mRNA levels by translation. Analysis of ADC1 and ADR2 transcription rates has not yet been performed.

Nuclease Sensitivity of ADC1 and ADR2 Chromatin

Transcriptionally active eukaryotic genes can be distinguished from inactive genes by their increased sensitivity to digestion with DNase I (20). The separation of ADC1 and ADR2 restriction enzyme fragments on agarose gels has allowed us to detect differences in their nuclease sensitivity that are correlated with transcriptional activity. Hereford and Lohr (16) reported that yeast chromatin was uniformly sensitive to DNase I. They did not assay any specific genes, however. We have investigated the nuclease sensitivity of ADC1 and ADR2 in nuclei isolated from cells

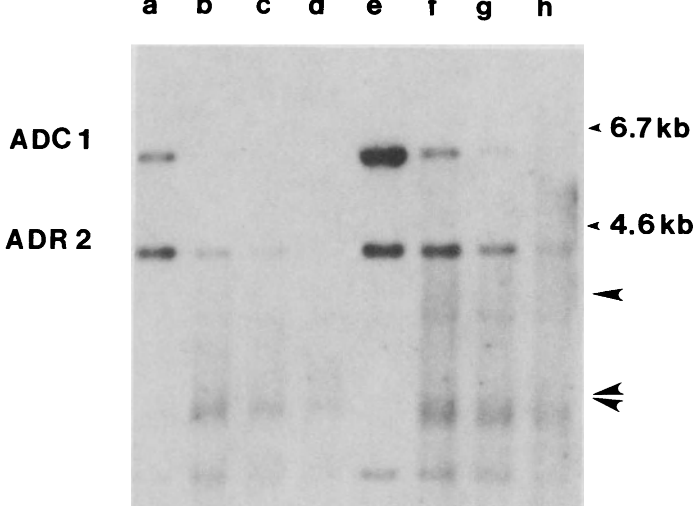

Figure 6. Hybridization of mixed ADC1 and ADR2 DNA to a Southern blot containing samples from DNase I digested yeast nuclei. Each lane of a 0.7% agarose gel was loaded with 3 µg of DNA. Lanes a-d contain DNA from nuclei isolated from yeast grown on ethanol (derepressed conditions), lanes e-h contain DNA from nuclei isolated from yeast grown on glucose (repressed conditions). After DNase I digestions all samples were digested with EcoRI. Lanes : a and e - only EcoRI digestion (control), b and f - nuclei digested with 1 unit of DNase I, c and g - 3 units of DNase I and d and h - 5 units of DNase I. The cloned DNAs used as probes were the 3.4 kb Bam H1 fragment from pJD14 for ADC1 and the 2.2 kb Bam H1 fragment from pADR2-BSa for ADR2.

growing in glucose (repressed ADR2) or ethanol (derepressed ADR2) containing media. The sensitivity to DNase I was assayed by isolating DNA from the treated nuclei, digesting it with a restriction enzyme, and following the degradation of the ADC1 and ADR2 restriction enzyme fragments by Southern transfer analysis using a mixture of ADC1 and ADR2 radioactive DNA as probes. The results are shown in Figures 6 and 7. In glucose-grown cells, the ADC1 gene is more sensitive than the ADR2 gene but in cells grown in ethanol the two genes are equally sensitive. There is a good correlation between the presence of ADR2 mRNA and the sensitivity of the ADR2 gene in chromatin. The ADC1 gene is equally sensitive in the cells grown in glucose or ethanol despite the decreased amount of ADC1 mRNA in cells grown on ethanol. In other systems studied there is no correlation between the extent of transcription and nuclease sensitivity; genes transcribed at a low rate are as sensitive as genes transcribed at a high rate (13). The same explanation might be true for ADC1 in cells grown on glucose and

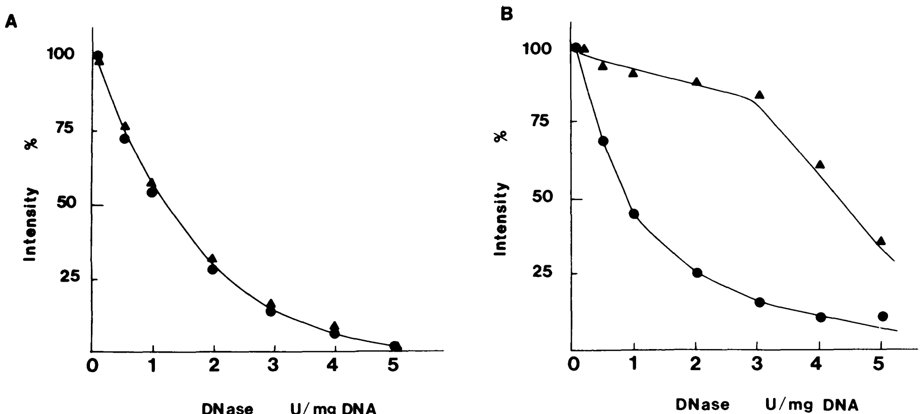

Figure 7. Sensitivity of ADC1 and ADR2 regions to DNase I in yeast growing on ethanol (A) and glucose (B). Blots similar to the one shown in Fig. 6, except that different DNase I concentrations were used, were scanned with a densitometer and the area under the peaks was determined at each DNase I concentration. The data represent the average from three separate blots. Circles - ADC1 gene; triangles - ADR2 gene.

ethanol. Alternatively, the rate of ADC1 transcription might be the same in the two growth conditions but the rate of degradation could be higher in ethanol-grown cells, thus decreasing the amount of ADC1 mRNA in these conditions.

Minor bands of lower molecular weight ADC1 and/or ADR2 are generated during DNase I digestion of nuclei (Figure 7). These DNA fragments appear to represent DNase I cleavage at nuclease hypersensitive site(s). We have mapped these sites using ADC1 and ADR2 probes specific for the 5' and 3' ends of the two genes (Figure 8), following the general procedure of Wu (25). The additional bands are specific for the 5' ends of ADC1 and ADR2 (Figure 9). There are two hypersensitive sites near the 5' end of ADC1 and one hypersensitive site near 5' end of ADR2; their positions are shown in Figure 8. These sites are near the putative mRNA start sites and may represent regions of the chromatin relatively free of nucleosomes.

Regulation of ADR2 by ADR1

ADR1 is a positive regulatory gene whose function is required for ADH II activity. Defective alleles of ADR1 and alleles allowing constitutive expression of ADH II have been isolated. By measuring the levels of ADH II enzyme activity, protein, and functional mRNA we showed that ADR1 was required for synthesis of functional ADH II mRNA, and that a constitutive mutant, ADR1-5^{c}

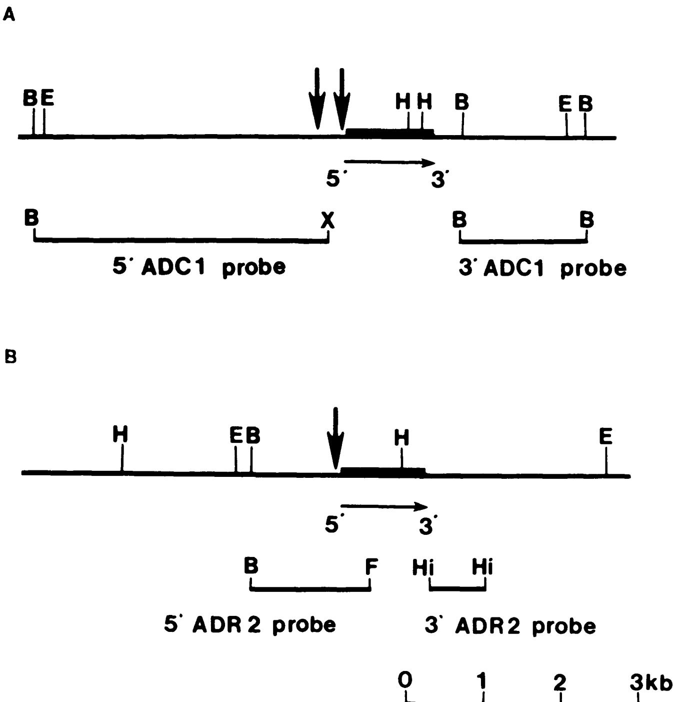

Figure 8. Partial restriction enzyme maps of the (A) ADC1 and (B) ADR2 regions. The positions of the cleavage sites of restriction nucleases was previously determined. Designations are as follows: B - BamHI; E - EcoRI; H - Hind III; X - XhoI; F - Fnu 4HI; Hi - Hinf I. Heavy line - structural ADH genes; horizontal arrows show direction of transcription and vertical arrows show the DNase I hypersensitive sites.

contained higher than normal levels of ADH II protein and mRNA (9). The constitutive mutant contained about 1/5 the derepressed ADH II mRNA level during growth on glucose-containing medium and increased about 10-fold when the culture was grown on ethanol-containing medium. The time course and extent of derepression of ADH II mRNA was very similar to that observed in a strain containing the wild-type ADR1 gene, indicating that the ADR1-5^{c} mutant was still subject to the phenomenon of catabolite repression.

We have confirmed the effect of ADR1 mutant alleles on ADR2 mRNA levels by performing quantitative S1 analysis as described in the previous section. The same RNA samples which had been assayed for functional ADH II mRNA were hybridized to a radioactive, single-stranded DNA probe containing the ADR2 5' end and

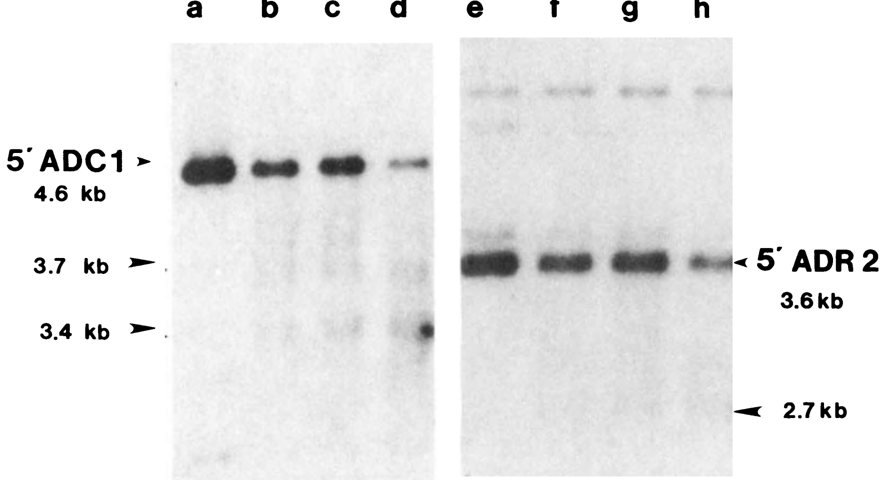

Figure 9. Mapping the DNase I hypersensitive sites adjacent to 5' ends of ADC1 and ADR2 genes. Lanes a to d - hybridization of 5' ADC1 probe and lanes e to h - hybridization of 5' ADR2 probe to a Southern blot containing samples from DNase I digested yeast nuclei (see Fig. 1 for localization of probes). Lanes a-d - nuclei were digested with DNase I (0; 0.5 U; 2 U; 4U) and EcoRI, lanes e-h - nuclei were digested with DNase I (0; o.5 U; 2 U; 4 U) and Hind III. Because the 5' ADR2 probe still contained some of the ADR2 structural sequences more bands appeared due to crosshybridization of the probe to ADC1 and ADM loci.

flanking sequences. The hybrids were digested with S1 nuclease. The samples were run on polyacrylamide gels and quantitated by densitometry (data not shown). The defective adr1-1 allele causes a 7-fold reduction in ADR2 mRNA relative to $ADR1^+$ (derepressing conditions). The $ADR1\text{-}5^c$ sample has a significant amount of ADR2 mRNA even when the RNA was isolated from glucose-grown cells. RNA isolated during derepression from cells containing $ADR1\text{-}5^c$ contain more ADR-2 mRNA than RNA isolated from $ADR1^+$ cells.

Altered Regulation of ADR2 by Transposable Elements

Seven out of a total of nine ADH II constitutive mutants at the ADR3 locus arose by the insertion of three distinctly different Ty elements (22). The relative level of constitutive enzyme activity, and the extent of derepression differed markedly for the different Ty-ADR2 constitutives (22). Two constitutive mutants, $ADR3\text{-}4^c$ and $ADR3\text{-}5^c$, do not contain Ty insertions. They differ from the Ty-ADR2 constitutives in that they have super-derepressed ADH II levels and are revertible to wild-type ADR2 phenotype.

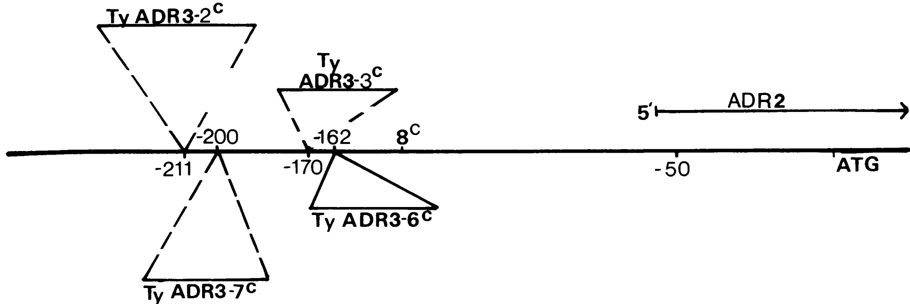

Figure 10. Ty insertions in ADR3. The position of five different Ty insertions are indicated.

A search for revertants of the Ty-ADR2 constitutive mutants yielded only an ADH II⁻ phenotype (7), most frequently as a result of excision of the Ty element but also by secondary mutations at unlinked recessive loci. A residual delta sequence (the direct repeat at the ends of the Ty element) remains after the Ty excision and is apparently responsible for the lack of ADH II activity. Insertion of a Ty element 5' to the his4C locus inactivates that gene and loss of the Ty allows gene expression to occur despite the continued presence of a delta sequence (11). Thus, opposite effects can occur by insertion of a Ty element adjacent to a gene.

We have cloned the Ty-ADR2 constitutive loci from five different $ADR3^c$ mutant strains. The ADR3 alleles cloned are 2^c, 3^c, 6^c, 7^c, and 8^c (6,22). The precise site of insertion of the Ty elements was determined by DNA sequencing. A schematic view of the sites of insertion, the 5' ends of the mRNA and the initiation codon of ADR2 are shown in Figure 10. The nucleotide sequence around the **sites** of insertion and the 5-bp duplications created at these sites (11,12) are shown in Figure 11. There is no striking sequence or base composition similarity at the sites of insertion. This whole region is AT rich as is most of the yeast genome. There

```
-250      -240      -230
GAGAATGAAAAAAAAAAAAAAAAAAAAAGGC

-220      -210        -200       -190      -180      -170        -160       -150      -140
AGAGGAGAG CATAG AAATGG GGTTC ACTTTTTGGTAAAGCTATAGCATGC CTATC ACA TATAA ATAGAGTATCCAGCTAGC

-130
GACTTTTTCACACTCG
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Figure 11. Nucleotide sequence of ADR3 region containing Ty insertions. The sites of Ty insertions are indicated by underlining of the sequence duplicated by insertion. Distances are given in nucleotide base pairs from the ATG initiation codon of ADR2.

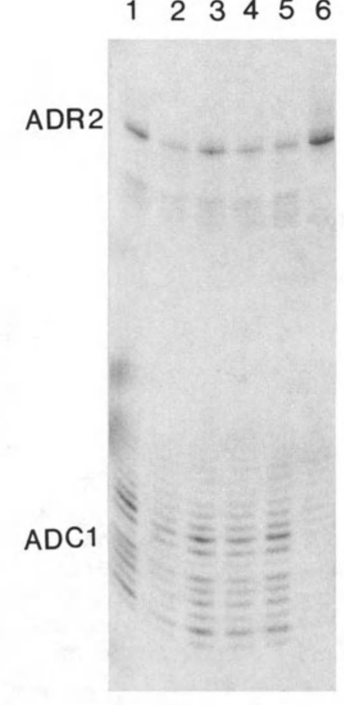

Figure 12. Determination of the 5' ends of ADR2 mRNA from wild type and mutant strains. SI mapping as described in Figure 3 was used to determine the 5' ends of ADR2 mRNA from various yeast strains: lane 4, an ADR3-7^{c}strain; lane 5, an ADR3-8^{c} strain; and lane 6, a wild type strain. RNA was extracted from cells grown with glucose as a carbon source for lanes 1-5 or from cells grown with ethanol as carbon source for lane 6. The larger number of bands in this figure is mainly due to DNA secondary structure since this experiment was done with excess DNA to allow quantitation of mRNA levels.

is a long poly dA stretch 5' to the sites of insertion but its significance, if any, is unknown. The Ty insertion in ADR-6^{c} duplicates the Goldberg Hogness "TATAA box" while the Ty insertions in ADR3-8^{c} occurs between the TATAA box and the ADR2 mRNA 5' end. Both of these insertions allow ADR2 transcription to occur with initiation at the normal site (see below).

One explanation for the constitutivity of ADH II after insertion of Ty elements is that the Ty element provides a glucose-insensitive promoter for ADR2 transciption. We have performed S1 mapping of the ADR2 transcripts present in the ADR3^{c} strains, as described in previous sections. These analyses are shown in

Figure 12. The same mRNA 5' ends are detected in RNA isolated from the parental, wild-type ADR3$^+$ strain and in the seven mutant ADR3^c strains. This is true even for ADR3-8^c in which the Ty insertion occurs between the TATAA sequence and the mRNA start sites at positions -55 and -57. These results suggest that either the mRNA 5' end we have mapped is a processed 5' end and that transcription in the ADR3^c mutants does proceed from the Ty element into ADR2 or that transcription at the normal ADR2 start site is activated by the inserted Ty element. The direction of transcription for the major Ty transcripts is away from the ADR2 gene (R. Davis, pers. comm.). This result makes the second alternative more likely.

The Ty activation of normally repressed ADR2 would occur if the Ty element interrupted a repressor binding site. However, this explanation is unlikely in view of the observation that when Ty excision occurs and a delta sequence is left behind, an ADH$^-$ phenotype results (7). A third possibility is that the Ty element contains in its epsilon region a site that activates the normal ADR2 transcription start site. This activation could be sensitive or insensitive to catabolite repression depending on the site or sites at which this system operates. That is, the Ty insertion may affect a site at which normal ADR2 regulatory proteins interact (such as ADR1 and the catabolite repression system) but Ty activation would be partially epistatic to these interactions depending on the site of insertion. It appears that the site of insertion is more important than the type of Ty element in determining gene activity since the same Ty element occurs in different locations in the ADR3^c mutants and these mutants have different levels of ADH II (22).

Effect of Mating Competence on ADH II Constitutivity

Ciriacy observed an apparent depression of ADH II activity in diploids heterozygous at the ADR3 locus (6) as compared to the constitutive activity in haploid cells. The expression of the ADR2 locus linked to an ADR3^c mutation was reduced in derepressed diploids whereas the wild-type locus was expressed normally. Subsequently, Sherman and collaborators (10) showed that CYC7-H2, a mutation that results in overproduction of iso-2-cytochrome c, was similarly reduced or abolished in diploids. The overproduction depended not on the ploidy of the cells but on the mating competence; a, α, a/a, or α/α cells all overproduced iso-2-cytochrome c but a/α cells and certain sterile haploid strains did not. The CYC7-H2 mutation is due to insertion of a Ty element very similar to the Ty elements found in ADR3-6^c and 7^c (10,22).

We have constructed homozygous diploids of the type ADR3-N^c ADR2$^+$/ADR3-N^c ADR2$^+$ and compared their ADH activity to the haploid

TABLE V
a/α Suppression of Yeast Alcohol Dehydrogenase Constitutive Mutations

ADR3 allele	Diploid Mat genotype	Specific activity:haploid/diploid	
		Medium containing	
		Glycerol	Glucose
ADR3$^+$	a/α	1	1.0
ADR3-1^c	a/α	11	2.5
ADR3-2^c	a/α	16	2.0
ADR3-3^c	a/α	19	2.3
ADR3-4^c	a/α	1	1.2
ADR3-6^c	a/α	65	2.0
ADR3-7^c	a/α	36	1.7
ADR3-8^c	a/α	38	1.5
ADR3-9^c	a/α	46	1.9
ADR3-2^c	a/a and α/α	1	1.3
ADR3-4^c	a/a and α/α	1	1.6
ADR3-6^c	a/a and α/α	1	1.3

Cells were grown overnight in media containing either gylcerol or glucose and were serially transferred twice to fresh media containing the same carbon source to insure balanced logarithmic growth until harvesting. Enzyme specific activities were measured at 25^o. The values represent the averages of two to four strains with the relevant genotype.

parental strains. The strains were tested after growth in either glucose (repression) or glycerol (derepression). The results are presented in Table V. In derepression conditions all of the Ty-linked ADR2 diploids have greatly decreased ADH II activity. The extent of derepression in the diploids ranges from 10-to 60-fold. This effect is unique to the Ty-linked ADR2 diploids. With ADR3-4^c, a putative point mutation in ADR3, ADH activity is not decreased in the diploid nor is the ADH activity decreased in strains with the wild-type ADR3$^+$ locus.

The depressed ADH II activity in diploids homozygous for Ty-linked ADR2 genes is not due to the ploidy of the cells. If the diploids are made homozygous for the mating type locus, the depression is greatly reduced so that enzyme activities in a/a or α/α diploids are similar to those in the haploid strains (Table V bottom).

The depression of ADH II activity in Ty-ADR2 diploids is very strongly affected by the carbon source in the growth medium. As shown in Table V, comparison of haploid and diploid ADH activities reveal at most a 2.5 fold depression in the diploids when the cells are grown in glucose-containing medium. This shows that Ty activation is sensitive to the carbon source in the growth medium as well as to mating competence.

DISCUSSION

Cloning yeast ADH genes by transformation and complementation of function has allowed us to isolate and characterize ADC1 and ADR2, the two genes coding for the major yeast isozymes, ADH I and ADH II respectively. Utilizing the cloned ADR2 gene as a hybridization probe we have cloned six different mutant alleles of the regulatory locus, ADR3, which is tightly linked to ADR2. We have also cloned by complementation of function ADR1, a gene which codes for a positive regulator of ADR2 expression.

ADC1 and ADR2 are 90% conserved at the nucleotide sequence level and 95% conserved in amino acid sequence. Within the 1041 nucleotide coding region there is a continuous stretch of 162 nucleotides in which there are no differences between the two genes. This region contains a histidine residue that participates in catalysis and the four cysteine residues that bind the structural Zn^{++} atom in a hydrophobic pocket of the enzyme. These functions might act to conserve amino acid sequence homology but they would not require the nucleotide sequence conservation which is observed. No other open reading frame exists in this region which could code for a second protein. It seems likely that either the two genes have exchanged genetic information in this region in the not-too-distant past or that the nucleotide sequence itself plays a role in regulation of transcription or accurate translation.

Nucleotide sequence homology in the 5' flanking sequences of ADC1 and ADR2 is particularly apparent in two regions. In the region complementary to the 5' end of the mRNAs for ADC1 and ADR2 28 out of 31 base pairs are identical. Further 5' to the coding sequence a second region of homology exists. Sixteen out of eighteen nucleotides around the Goldberg-Hogness "TATAA" box are conserved. This sequence is thought to be important in transcription initiation in eukaryotes and is commonly found about 30 nucleotides 5' to the RNA start site. In ADC1 and ADR2 this sequence is about 100 nucleotides from the start site. The 5' mRNA ends that we have mapped using S1 analysis could be processed ends of a larger precursor RNA that we have failed to detect. If this is true, the actual start sites could be closer to the TATAA sequence. Parenthetically, it has not been proved that this sequence is essential for transcription in yeast.

The evidence that the glucose repressed isozyme, ADH II, is transcriptionally regulated was based initially on assaying the functional ADH II mRNA by *in vitro* translation (8). This result has been confirmed by using a cloned *ADR2* DNA as a hybridization probe and performing S1 nuclease analyses on RNA extracted from cells grown in media containing glucose or ethanol as a carbon source. Both *in vitro* translation and S1 analysis indicated that *ADC1* was regulated in an inverse fashion to *ADR2*. In cells grown in ethanol-containing medium, when *ADR2* mRNA is abundant, *ADC1* mRNA is decreased 5 to 10-fold relative to the amount present in cells grown in glucose-containing medium, when *ADR2* mRNA is at a very low level. Measurement of transcription rates by labelling RNA and hybridizing it to *ADC1* and *ADR2* DNA has not been attempted because of the homology between the two genes.

Transcriptionally active eukaryotic genes are characterized by increased sensitivity to nuclease digestion compared to non-transcribed genes. We have used this assay to confirm and extend the results presented above which indicate that *ADR2* is transcriptionally regulated. The *ADR2* gene in nuclei isolated from cells grown in glucose-containing medium is much more resistant to DNase I digestion than either the same gene in nuclei isolated from ethanol-grown cells, or the *ADC1* gene in nuclei isolated from cells grown with either carbon source. Thus the nuclease sensitivity of *ADR2* is correlated with the presence of *ADR2* mRNA whereas the nuclease sensitivity of *ADC1* is not correlated with the amount of *ADC1* mRNA. This difference could arise if *ADR2* is not transcribed at a significant rate in cells growing on glucose as a carbon source whereas *ADC1* continues to be transcribed in cells growing on ethanol, albeit at a reduced rate.

Sites hypersensitive to nuclease digestion have been located 5' to both *ADC1* and *ADR2*. These sites are in the transcription initiation regions.

As one approach to determining which nucleotides in the 5' flanking regions are important for transcription of *ADC1* and its regulation we have constructed deletion mutants *in vitro*. These deletions extend different distances into the 5' flanking regions from a restriction enzyme site located 260 base pairs from the initiation codon. Plasmids containing deletion mutations have been introduced into yeast lacking ADH activity so that the effect of synthesis and regulation of ADH I can be assayed. A deletion lacking the nucleotides 5' to position -70 (with respect to the initiation codon) yields no ADH activity, whereas a deletion lacking nucleotides 5' to position -150 yields a nearly normal level of ADHI activity. The TATAA box is at position -125. Whether it is this sequence or some other sequence between -70 and -150 that is essential for ADH expression is unknown at present.

A cis acting regulatory site closely linked to ADR2 was identified by Ciriacy (5). This region, ADR3, with the adjacent ADR2 gene has been cloned from six different $ADR3^c$ mutant strains which express ADH II constitutively. Five of these strains contain insertions of a yeast transposable element, Ty, at positions ranging from approximately 140 bp to 211 bp 5' to the initiation codon of ADR2. The sixth $ADR3^c$ mutant does not contain a Ty element.

The insertion of each Ty element results in a different 5 bp duplication of a genomic sequence at each end of the Ty element. No obvious sequence preference is shown for the site of insertion. In $ADR3\text{-}8^c$ the Ty insertion occurs between the TATAA sequence and the RNA start site. Either this sequence is not required for ADR2 transcription or a sequence with an analogous function to the normal TATAA sequence is present in the repeated delta sequence element.

The sites of insertion do not offer an obvious explanation for the differences in ADH II phenotype observed in the different $ADR3^c$ mutants. There is no simple correlation, for example, between the position of the Ty insertion and the level of ADH II activity. However, the site of insertion, and not the type of Ty element, seems to be the significant factor in causing these differences since the ADH activity of two mutants with similar Ty elements inserted in different positions differs considerably.

The mechanism by which Ty insertion causes ADR2 activation has been studied by measuring the size and identifying the 5' ends of the ADR2 mRNA isolated from the $ADR3^c$ mutant strains. Both types of experiment indicate that the ADR2 mRNAs synthesized due to Ty activation are identical to the ADR2 mRNA made during derepression in a wild-type $ADR3^c$ strain. Thus, transcription does not start in the Ty element and continue into the adjacent ADR2 gene unless the presumptive ADR2 initiation site is in fact an RNA processing site.

The activation of ADR2 by an adjacent Ty element is sensitive to mating competence, as has been observed for Ty activation of the CYC7-H2 locus (10). In a/α diploid cells, but not in a/a or α/α cells, the expression of Ty-linked ADR2 genes is drastically reduced compared to their expression in haploid strains. This depression is most evident during growth on a non-fermentable carbon source such as ethanol or glycerol.

In summary, the regulation of the ADH genes of Saccharomyces cerevisiae is subject to complex metabolic and genetic control. This makes these genes exceptionally interesting to study in order to learn more about eukaryotic gene regulation. From this knowledge will come the ability to modulate the expression of these genes to achieve more efficient production of ethanol and other chemicals.

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GENETIC DEREGULATION OF ETHANOL-RELATED GENES

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Wild-type *Escherichia coli* strains are unable to use ethanol as a carbon source. However, they do produce ethanol as a fermentation product under certain conditions of anaerobic growth. In the absence of nitrate, at acidic pH, and especially in the presence of high phosphate concentrations, *E. coli* ferments glucose mainly to lactic acid. However, at alkaline pH and in the absence of phosphate an equimolar mixture of acetate plus ethanol is the major product (11).

During experiments in which certain fatty acid derivatives were fed to acetate auxotrophs, we inadvertently isolated mutants that were able to use, as an acetate source, the small amounts of ethanol in which the fatty acid derivatives had been dissolved (4). The utilization of ethanol seemed likely to occur by reversal of the pathway whereby *E. coli* produces ethanol from acetyl-CoA under anaerobic conditions. Ethanol would first be converted to acetaldehyde by alcohol dehydrogenase (ADH) and the acetaldehyde would be oxidized to acetyl-CoA. We have confirmed the previous report (12) of ADH in *E. coli* and have demonstrated the presence of acetaldehyde CoA dehydrogenase (ACDH) in this organism. The pathway of ethanol utilization is:

$$C_2H_5OH + NAD \longrightarrow CH_3CHO + NADH_2 \qquad (1)$$

$$CH_3CHO + NAD + CoA \longrightarrow CH_3\text{-CO-SCoA} + NADH_2 \qquad (2)$$

Reaction 1 is catalysed by ADH and reaction 2 by ACDH.

This pathway is nonfunctional in aerobically growing wild type

E. coli cells. However, the mutants we isolated overproduce ADH and ACDH both aerobically and anaerobically.

1. Growth Properties and Enzyme Levels of adhC Strains

The adhC mutants were isolated as strains able to use ethanol to satisfy the acetate requirement engendered by a mutation in pyruvate dehydrogenase (4). The mutants fell into two classes, those utilizing ethanol only as an acetate source and those also able to use ethanol as a sole carbon source (4). Both types of adhC mutants overproduce ADH and ACDH. However, the degree of overproduction is greater in those strains utilizing ethanol as a carbon source. For example, one such strain, DC272, overproduces ADH >100-fold when grown aerobically in the absence of ethanol (Table I). We have concentrated our work on this type of strain.

The levels of ACDH follow a similar but quantitatively different pattern (5). The adhC mutants overproduce ACDH about 10-fold when grown aerobically (Table I, II) but addition of ethanol or acetaldehyde to the medium increases the ACDH level 2 to 4-fold (Table II). In contrast, addition of ethanol or acetaldehyde increases ADH only 1.5 to 2-fold. The adhC strains also overproduce ADH and ACDH when grown anaerobically. ADH is overproduced 100-fold whereas ACDH is overproduced only 3-fold (Table I).

It is clear that the overproduction of ADH and ACDH is responsible for utilization of ethanol since revertants of the adhC mutants can be isolated by screening on tetrazolium indicator plates containing ethanol (5). These revertants fall into three classes: i) lacking both ADH and ACDH, (revertant I, Table I) ii) lacking only ACDH (revertant II) and iii) retaining both enzymes. We have not yet isolated revertants lacking only ADH by this method although adhC strains lacking ADH but retaining overproduction of ACDH have been isolated by other means (see below).

2. Interrelationships with Other Regulatory Mechanisms

When grown anaerobically in the presence of nitrate as the terminal electron acceptor, E. coli does not produce ethanol or other reduced carbon compounds (11). This finding coupled with the fact that the adhC gene is located very close to chlC, a nitrate reductase structural gene (4), led us to examine the interplay of the ethanol and nitrate metabolic systems.

The adhC mutation represses the level of nitrate reductase 5 to 10-fold in anaerobically grown cells in the absence of nitrate (Table III). This repression is largely overcome by the addition

TABLE I

Enzyme Activities of adhC Mutants

Strain	Aerobic		Anaerobic	
	ADH	ACDH	ADH	ACDH
Wild type	0.1	1.9	3.3	55
adhC	119	16	554	177
Revertant I	4.1	0.5	--	--
Revertant II	88	0.5	--	--

Values are given are U/mg of extract protein. One unit of either enzyme is defined as 1 nmol of product formed per min at 22°C. The ADH level of the adhC strain grown anaerobically at pH 8 in the presence of 0.1% nitrate was 447 U/mg protein.

of nitrate. Conversely, the presence of nitrate represses production of ADH both in adhC mutants grown aerobically (Table III) and in wild type strains grown anaerobically (4).

The levels of ADH and ACDH are also affected by catabolite repression. The presence of glucose decreases the ADH and ACDH levels in the adhC mutants 3-fold in aerobically grown cells. The non-metabolizable glucose analog, α-methylglucoside, has no effect on ADH but does repress ACDH (Table II).

Genetic Characterization

Both the mutations lacking ACDH (called acd) and the adhC locus have been located on the E. coli genetic map. The adhC locus maps adjacent to the trp locus, very close (>95%) to the chlC gene of the nitrate reductase system (4). The acd mutants map at a site one-third of the genetic map removed from the adhC locus, between the lysA and serA loci (5). The adhC gene therfore controls the activities of enzymes controlled by genes either close to (chlC) or far removed (acd) from the adhC locus.

Diploid tests have shown that introduction of the wild type ($adhC^+$) allele into strains carrying the adhC allele resulted in no decrease in the high level of ADH characteristic of adhC strains (4). Thus $adhC^-$ is dominant over $adhC^+$.

TABLE II

Regulation of Enzyme Levels

(adhC grown aerobically)

Addition	Percent ADH	Percent ACDH
None	100	100
Nitrate (0.1%)	25	n.d.
Glucose (0.5%)	30	30
αMethyl Glucoside (10 mM)	100	25
Ethanol (0.5%) or Acetaldehyde (0.1)%	150	350

Values of 100 percent are 85 and 160 U/mg protein for ADH and ACDH, respectively. n.d. = not determined.

3. Regulation of Ethanol Utilization

Mutations at the adhC locus turn on the production of both ADH and ACDH and decrease the production of nitrate reductase. Although the adhC mutants were isolated as strains able to utilize ethanol aerobically, these strains also overproduce ADH anaerobically (4).

The adhC mutants are dominant over the wild type allele and thus are candidates for being promotor (or other cis acting) mutations rather than a classical repressor protein. These mutants affect both a closely linked gene, chlC, and an unlinked gene, acd. It is possible that the adhC locus overproduces a protein regulator of certain operons which are expressed anaerobically. However, at present our information is sufficiently scanty that other possibilities abound. For example, the effect on nitrate reductase could be indirect and have nothing to do with enzyme synthesis. The effect on ACDH could also be indirect. Perhaps the adhC locus is the promotor for the ADH gene and the action of ADH produces acetaldehyde which then induces ACDH.

Other possibilities come from our recent attempts (6) to isolate mutations in the ADH and ACDH structural genes using active site directed reagents (7). ADH converts allyl alcohol to the highly toxic alkylating agent, acryladehyde (CH_2-CH = CHO)(8). By selecting for resistance to allyl alcohol we have isolated mutants which lack

TABLE III

adhC Repression of Nitrate Reductase

	No NO_3	Plus NO_3
Wild type	8.2	390
adhC	1.1	200

The cells were grown anaerobically in rich broth plus Tris (pH 8.0) and glucose. For units see Table I.

both alcohol dehydrogenase and acetaldehyde CoA dehydrogenase activity under aerobic and anaerobic conditions (6). In addition, temperature sensitive mutants were found which contained a thermolabile alcohol dehydrogenase, indicating a lesion in the structural gene (6). Some, but not all, of these temperature sensitive mutants also contained a thermolabile acetaldehyde CoA dehydrogenase. All three types of mutant mapped very close to adhC locus (6). These curious results suggest that ADH and ACDH have some structural interaction and raise the possibility that ACDH is only active when complexed with ADH. This hypothesis would explain why revertants of adhC strains lose both ADH and ACDH or ACDH but not just ADH (Table I). Previous workers have reported that ACDH sediments as a large metastable complex (10) and that purified ACDH from E. coli B contains some ADH activity (9). These results suggest that a complex between ADH and ACDH exists. Such a complex might be a safety mechanism to prevent, during ethanol formation, accumulation of acetaldehyde, a highly reactive and toxic molecule. The requirement for complexation with ADH for function of ACDH would ensure that acetaldehyde is produced only when it can be quickly reduced to ethanol.

Other preliminary work indicates that the levels of ADH are regulated by transcription (2). Using the Mu dlac fusion phage of Casadaban and Cohen (1), we have isolated strains resistant to both ampicillin and allyl alcohol that synthesize β-galactosidase anaerobically but not aerobically. These mutants map at the adh locus and thus are probably fusions of the adh promotor to the lacZ gene. We have also isolated a number of other Mu dlac insertions that synthesize β-galactosidase under anaerobic but not aerobic conditions. It, therefore, seems that transcriptional regulation is a major mechanism in the adaption of E. coli to anaerobiosis.

Can E. coli be Used for Ethanol Production?

Unfortunately, for the purposes of this conference, we have studied ethanol utilization rather than ethanol production. However, the same pathway is used and thus we would expect the adhC strains to overproduce ethanol when grown anaerobically. E. coli is fairly tolerant to growth in the presence of ethanol (3). We have strains that tolerate 6% ethanol at 37°C and it should be possible to isolate more tolerant strains, perhaps by manipulation of the cellular lipid composition (3).

E. coli can grown on a wide variety of carbon sources. An interesting possibility is that growth on xylans, a major byproduct of wood processing, might be possible. E. coli grows well on xylose and this carbon source exerts only minimal catabolite repression and thus should not effect ADH and ACDH levels.

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LACTATE DEHYDROGENASE-DEFICIENT MUTANTS OF *STREPTOCOCCUS MUTANS*

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INTRODUCTION

One approach to the prevention of bacterial infections had its formal beginning over a centruy ago. Pasteur, along with his colleague Joubert, noted that the growth of anthrax bacillus in a urine specimen was inhibited by the presence of other "common" microbes (15). They predicted that the infection of a susceptible host by certain harmless bacteria might prevent subsequent infection by virulent types of bacteria. The acutal mechanism underlying this approach, which has recently been termed *replacement therapy*, would depend on the ability of the harmless, so-called *effector strain* to occupy the niche in the susceptible host tissues normally occupied by the pathogen. Once well established there, the effector strain could follow one of several courses to prevent infection by a particular pathogen.

Although the human oral cavity harbors a large variety of bacteria, there is considerable evidence to implicate a particular species, *Streptococcus mutans*, as the major cause of dental caries (5,12,14). The principal features of this bacterium that predispose it to cause decay include its ability to stick to teeth (6,8) and its ability to produce large amounts of lactic acid from the metabolism of sugars (10,17). It is this acid that appears to be directly involved in the decay procees by reducing the pH in the microenvironment of the tooth surface below a threshhold level, and thus promoting dissolution of the mineral phase of enamel and dentin (9). Eventually, cavitation of the weakened surface occurs, resulting in clinically observable caries.

Given these facts, one choice for an idealized effector strain

for use in the replacement therapy of dental caries might be a genetically altered strain of S. mutans, which is similar to its parent in every regard but for a decreased ability to make acid from the metabolism of carbohydrates. Such a strain would be expected to occupy the niche in the oral cavity normally occupied by naturally occurring strains of S. mutans but would itself be less pathogenic.

Isolation and Preliminary Characterization of LDH-Deficient Mutants.

Several years ago, a mutant strain of S. mutans was isolated which seemed to fit this description. While the parental strain BHT-2 (serogroup b) produced small white colonies when incubated in candle jars on Lederberg's glucose tetrazolium medium (13), mutagenesis with ethylmethane sulfonate led to the generation of rare red colonies which were observed amidst a background of white, wild-type appearing colonies. Several of these isolated red colonies appeared to be pleiotropically affected, producing red colonies on tetrazolium medium when glucose, fructose, mannitol, or lactose served as the carbon source.

These mutants were significantly less effective in reducing the pH of culture medium during 48 hours of incubation in Todd-Hewitt broth containing 1% glucose than was their parent, BHT-2 (Table I). The difference observed did not reflect a difference in the amount of glucose consumed. Furthermore, neither growth rates nor cell yields differed significantly. Culture liquors of the mutant strains contained no detectable lactic acid when analyzed by gas-liquid chromatographic methods (16). On the basis of this phenotype it seemed likely that the mutants were deficient in the L(+)-lactate dehydrogenase (LDH) activity which had previously been described in S. mutans (3). Crude cell-free extracts of these strains were indeed found to contain ca. 1% of the parental level of this activity. Mixed wild-type and mutant extracts gave additive activities.

A total of 18 independent mutants possessing these phenotypic properties have been isolated from strain BHT-2. Temperature sensitive revertants of several of these strains have been shown to possess a thermolabile LDH activity suggesting that the genetic lesions are in the structural gene for the enzyme. Rabbit antiserum prepared against LDH purified from S. mutans was used to screen crude cell-free extracts of the mutants for deletion and nonsense mutations. No such mutations were identified by these methods.

Fermentation Properties

Fermentation of glucose by washed cell suspensions of BHT-2 and an LDH-deficient mutant, JH140, were carried out at pH 4,5,6,

TABLE I
Growth Properties of BHT-2 and LDH-Deficient Mutants*

Strain	Terminal pH	Cell Yield (OD_{580})	Glucose Consumed (mol/ml)	Lactic Acid Produced (mol/ml)
BHT-2	4.38	2.4	47.2	33.9
LDH^-	4.80	2.4	45.6	... 1.0

* Strains were subcultured 1:100 into Todd-Hewitt broth containing 1% glucose. After 48 h of incubation in candle jars at 37°C, the absorbance at 580 nm (OD_{580}) and pH of the cultures were determined. Lactic acid production and glucose consumption was determined by gas-liquid chromatography (16) and glucose oxidase assays, respectively, of the culture liquors.

and 7. The method employed glucose grown overnight cultures which had been washed and concentrated 10-fold in 1 mM potassium phosphate buffer. Following addition of glucose to the cell suspension, the rate of acid production was monitored by determining the amount of N NaOH required to maintain the pH at the predetermined level. At the indicated times, samples were removed to determine the amounts of glucose which had been consumed (Figure 1).

As seen if Figure 1, the amount of glucose consumed in order to reach plateau was about the same for both the parent and mutant at the three pH conditions examined. However, the ratio of acid produced to glucose consumed differed significantly between the strains. At pH 6 and 7, the data obtained for BHT-2 approached the theoretical value of 2 for a homolactic fermentation. At pH 5, this value dropped somewhat, suggesting a (partial) shift to a less acidogenic pyruvate-degrading pathway. The values for the mutant decreased significantly with each successive decrease in pH and were ca. 80, 70, and 60% of the parental values at pH 7,6, and 5, respectively. At pH 4.0 the reaction performed by the parental strain proceeded at a rate too slow to obtain meaningful data. At this pH the reaction using the mutant strain did not proceed, in agreement with the earlier finding (Table I) that 4.8 was the lowest pH attained by a growing culture. In contrast to the parent strain, JH140 showed a lag in glucose consumption and acid production which became more pronounced as the pH of the fermentation reaction decreased. This finding correlates with a variably longer lag phase observed for the mutant compared to the parent when subculturing in glucose-containing broth.

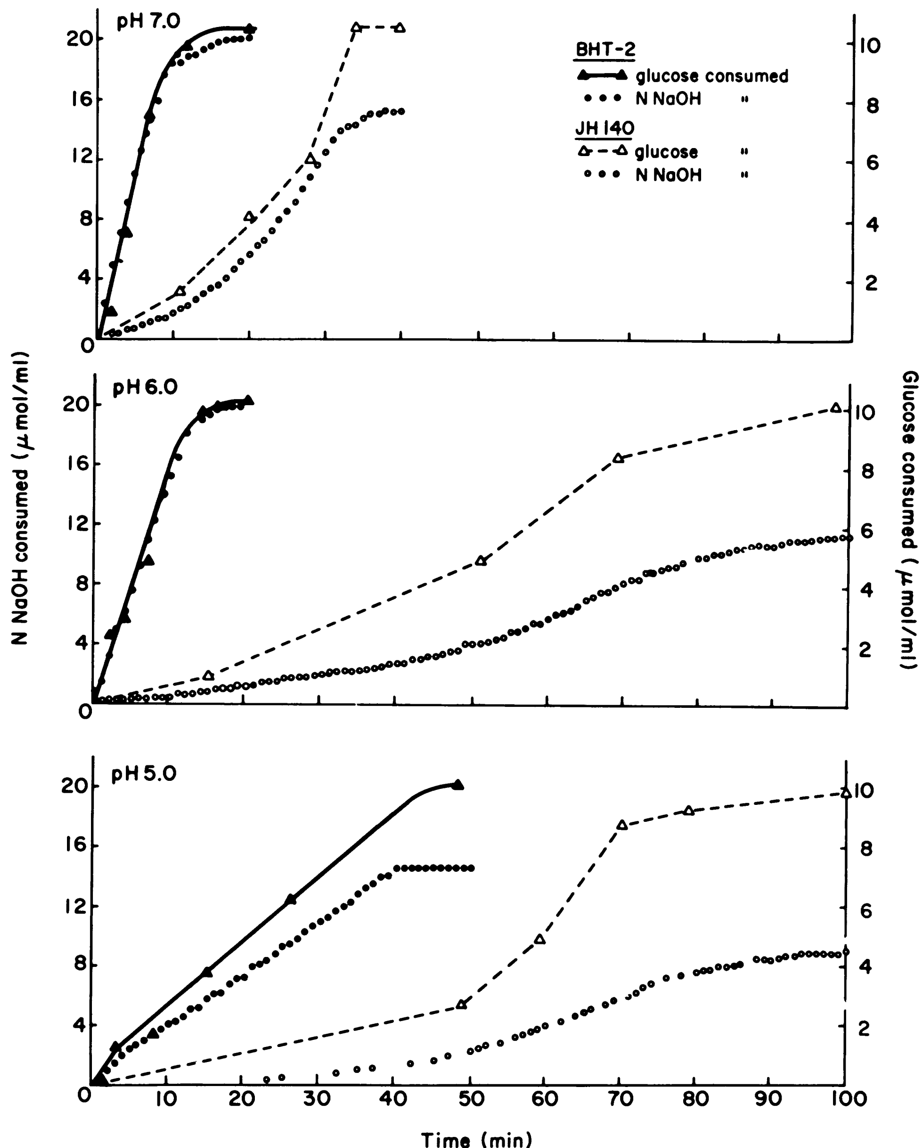

Figure 1. Kinetics of glucose fermentation by wild-type and LDH-deficient strains. Washed, resting cell suspensions of BHT-2 and JH140 were incubated with 0.2% glucose at 37°C in 1mM potassium phosphate buffer. The suspensions were maintained at the indicated pH by automatic addition of N NaOH. At the indicated times, samples were removed to determine the amount of glucose consumed.

Qualitative analyses of broth cultures of the mutants revealed formate, acetate, ethanol, acetoin, and trace amounts of diacetyl as the principal end products of glucose fermentations. These findings accord with studies showing that various so-called homolatic acid bacteria, including S. mutans, possess alternate pathways for pyruvate dissimilation that seem to function at significant levels only under certain conditions of growth. For example S. mutans, when grown under conditions of glucose limitation (4) or when grown on a relatively poor carbon source such as mannitol (2), produces significant amounts of formate, acetate, and ethanol.

Of interest is the fact that attempts to isolate LDH-deficient derivatives from other laboratory strains of S. mutans have to date been unsuccessful. This is of concern since serogroup c strains tend to predominate in human populations while serogroup b strains, such as BHT-2, are most frequently isolated from rodents. Recently, we have noted that aerobically grown glucose-broth cultures of BHT-2 contain 2 to 3 fold more formate and acetate than do culture liquors of most serogroup c strains. BHT-2 also distinguishes itself from most serogroup c strains on Lederberg's glucose tetrazolium medium in which 2,3,5-triphenyl tetrazolium is replaced by neotetrazolium. Under these conditions BHT-2 produces deep blue colonies compared to the pink or light blue colonies of serogroup c strains. Mutants of serogroup c strains OMZ 176 have been obtained that produce relatively dark blue colonies on neotetrazolium medium. Compared to their parent, culture liquors of these mutants appear to contain increased amounts of formate and acetate. Thus, we currently speculate that BHT-2 has a fortuitous mutation rendering the biosynthesis and/or activity of its pyruvate-formate lyase system insensitive to the inhibitory effects of oxygen (20). BHT-2 would, thus, have an alternate pyruvate dissimilating pathway available to it under the aerobic conditions employed to screen for LDH-deficient mutants.

Aside from the above-described differences in their acid producing properties, the LDH deficient mutants of BHT-2 appear identical to their parent in every regard. In particular, they can still stick to teeth and form plaque, and they retain the same nutritional requirements as their parent. This is precisely the set of properties that we predicted an idealized effector strain must have in order to serve in the replacement therapy of dental caries. To directly test the ability of the mutants to behave as effector strains, their cariogenic potential and ability to prevent infection by wild-type S. mutans was examined both in the laboratory and in animal models.

Cariogenic Potential

In brief, we found that when human teeth were immersed in sucrose-containing cultures of BHT-2, and fresh medium was added

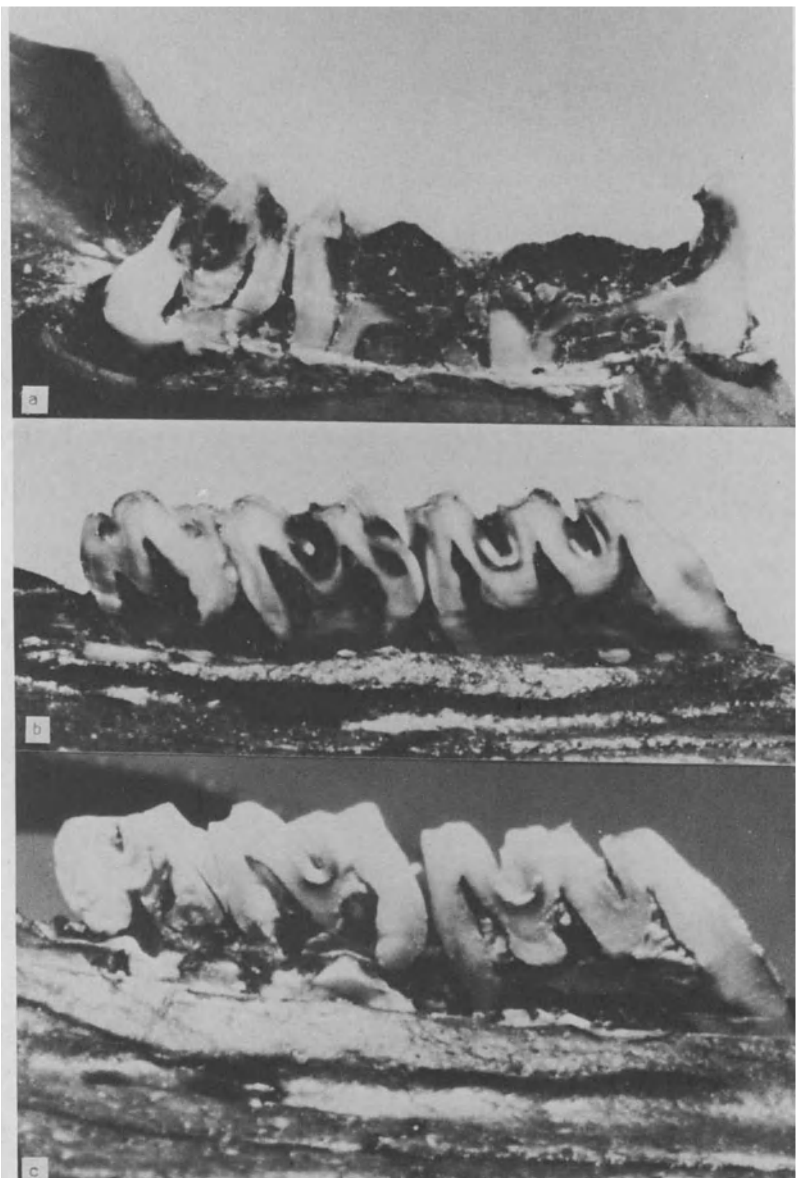

Figure 2. Cariogenicity of parent and LDH-deficient strains in conventional rats. After 14 weeks of infection, the molar teeth of conventional Sprague-Dawley rats infected with BHT-2(a), JH145 (ldh)(b), or sham-infected (c) were sagitally sectioned and stained for areas of demineralization.

daily, incipient carious lesions called white spots developed after only 10 days of incubation. In contrast, human teeth exposed to cultures of an LDH-deficient mutant were free of visible pathology even after 21 days of incubation.

The pathogenic potential of the mutants was also tested in a more direct fashion using germfree and conventional, pathogen-free Sprague-Dawley rats. In these experiments, separate groups of animals were infected with BHT-2, an LDH-deficient mutant, or sham-infected. After 14 weeks of infection, during which the rats were fed a high sugar diet to promote decay, the teeth of the animals were examined. As can be seen in Figure 2, conventional rats infected with the LDH-deficient mutant exhibited an incidence and severity of carious lesions comparable to the sham-infected control group. Both groups developed significantly fewer and less extensive lesions than did BHT-2 infected animals. Since it could be shown that the mutant populated the oral cavity of the rats to the same extent as BHT-2, the observed difference in cariogenic potential could be attributed solely to the difference in their acid producing characteristics. On the basis of these and other experiments, LDH-deficient mutants appear to satisfy one of the two basic requirements for use as an effector strain in replacement therapy: that is, because they produce less acid from the metabolism of sugar than do naturally occurring strains of *S. mutans*, they are virtually noncariogenic.

Competitive Properties

The basis for determining the ability of the mutants to satisfy the second prerequisite for use as an effector strain, namely their ability to colonize the host and thereby prevent infection by naturally occurring strains of *S. mutans*, is conceptually somewhat more complicated. In brief, there are two important observations to consider. First, as with other bacterial infections, persistent colonization of the oral cavity of *S. mutans* appears to require exposure to a threshhold concentration of cells, the so-called minimal infection dose [MID; (19)]. Second, once having established itself as a part of the oral flora, a resident strain of *S. mutans* is relatively resistent to displacement or superinfection by a challenge strain of *S. mutans* (11). These observations can be explained by presuming that the MID for persistent colonization of *S. mutans* reflects both the probability that a cell introduced into the mouth will find an available attachment site on a tooth surface and the probability that once attached it will compete favorably with the resident flora for essential nutrients. It follows that the establishment of one strain of *S. mutans* in the oral cavity should inhibit subsequent colonization by a superinfecting strain by reducing the chance that both of these requirements would be met. This situation would be reflected in the

occurrence of a higher MID in order to achieve persistent colonization by a superinfecting strain.

In our studies with LDH mutants, we have found that conventional rats which have a complex oral flora but which are initially free of S. mutans can become infected by various strains of wild-type S. mutans when challenged with an inoculum containing approximately 1×10^5 cells. In accord with our prediction, we have found that if the animals are first infected with an LDH-deficient mutant, the MID for infection by a wild-type strain of S. mutans is increased to 10^7 or 10^8 cells.

At the present time it is not known whether LDH mutants will exert a similar effect in the human oral cavity. Indeed, details of the mechanism(s) whereby S. mutans gains access to and colonizes the oral cavity of humans are, as yet, unclear. From what is known of the ecology of S. mutans, it seems likely that colonization of an uninfected individual occurs at an early age by contact with other humans (1). Certainly, exposure to contaminated saliva where concentrations of S. mutans may reach levels of 10^6 cells per milliliter (18), is a logical but unproven route of transmission. It is therefore impossible to say for certain whether the presence of the mutant on human teeth will prevent the natural transmission of wild-type strains of S. mutans. Clearly, human clinical trials will be required to answer this question.

CONCLUSION

The principal approaches which are currently in use to control the incidence and severity of dental caries have involved the restriction of sugar consumption and the application of fluorides and good oral hygiene. Owing to their incomplete effectiveness and the difficulty of educating the public as to their value, these several approaches have been found to be inadequate (7). Since dental caries is a disease known to be caused by bacteria, antibacterial agents such as antibiotics and disinfectants and, more recently, vaccination against S. mutans, have been extensively studied. In general, these several approaches suffer from at least one common drawback: the likelihood of inducing the outgrowth of genetic variants which are no longer susceptible to the agent. From a theoretical standpoint, the use of replacement therapy, as described here, provides a seemingly ideal approach to the control of dental caries. The competitive, non-pathogenic effector strain could be introduced into the mouths of subjects following elimination or suppression of indigenous strains of S. mutans (by short term antibiotic or disinfectant therapy), or preferably into the mouths of children prior to their acquisition of a naturally occurring strain. Once established, the effector strain could conceivably provide lifelong protection against the disease while requiring very little

in the way of cooperative assistance, expense, or education on the part of the patient. This approach has the possible added advantage of providing so-called 'herd protection' by the natural transmission of the effector strain within the population. Finally, it should be emphasized that most any bacterial or viral infection should be amendable to control by this approach. The application of these methods does, however, require a basic knowledge of the identity, ecology, and pathogenic mechanisms of the infectious microorganism. Once these have been established, as in the case of S. mutans, a methodical search for a useful effector strain can be undertaken to control the incidence and severity of the disease.

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DISCUSSION

Q. Why do some people lack tooth dacay?

A. The propensity of an individual to develop tooth decay depends on a variety of factors, the most important of which seem to be the presence or absence of Streptococcus mutans on the teeth, dietary habits, and fluoride experience. The rare individual who reaches adulthood free of tooth decay thus may lack Strep. mutans from his or her oral flora, may have a low and infrequent consumption of dietary sucrose, or may have a high level of fluoride incorporated into the mineral phase of enamel.

Q. Could you account for all the glucose utilized stoichiometrically in terms of the produce formation in case of the mutant since there was no increase in cellular synthesis but drastic decrease in lactic acid formation?

A. Accurate carbon and redox balances are currently being performed for glucose fermentation by the LDH-deficient mutants. Preliminary data suggests that formate, acetate, ethanol, acetoin, diacetyl, and CO_2 will account for virtually 100% of the carbon.

Q. When you introduce both the mutant and wild type S. mutans into the oral cavity, does one dominate growth over the other?

A. Simultaneous infection of germfree rats with BHT-2 and and LDH-deficient mutant leads to a stable mixed infection. This is not surprising since current evidence suggests that on many areas of the tooth surface, particularly the smooth surfaces, Strep. mutans exists as discrete micro-colonies. Such micro-colonies, depending on the degree of their physical isolation, might not influence each other to any great degree.

GENERATION OF PRODUCTS BY METHANOTROPHS

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NATURE OF METHANOTROPHIC BIOTRANSFORMATION ACTIVITIES

It has been recognized for many years that obligate methanotrophs, although requiring C_1 compounds for growth, are nevertheless capable of effecting the partial oxidation of several simple methane analogues (short chain alkanes and alkenes) to products which accumulate extracellularly (64). Indeed, the work of Foster and his colleagues (29,30) represents one of the early classical examples of cooxidation. It has generally been assumed that these oxidations are a result of the lack of specificity of enzymes involved in the oxidation of methane to carbon dioxide. The findings of Dalton and his colleagues (7,49) that partially purified methane monooxygenase (MMO) preparations oxygenate a wide range of organic compounds, supported this hypothesis. The extraordinary wide range of oxidations catalyzed by this enzyme in some species is, however, surprising; in addition to alkanes and alkenes, aromatic, alicyclic and heterocyclic compounds are oxidized.

The route of oxidation of methane by methanotrophs is shown in Figure 1. Enzyme 2, which catalyzes the further oxidation of methanol, methanol dehydrogenase, is also relatively non-specific, (36-38, 58) as are some aldehyde dehydrogenases in some obligate methanotrophs (1,50). In addition, a non-specific secondary alcohol dehydrogenase has been found in several species (25) and this enzyme has no known function in C_1 metabolism.

Enzymes involved in biotransformation reactions.

$$CH_4 \xrightarrow{1} CH_3OH \xrightarrow{2} HCHO \xrightarrow{3} HCOOH \xrightarrow{4} CO_2$$

Step	Enzyme
1	Methane monooxygenase
2	Methanol dehydrogenase
3	Formaldehyde dehydrogenase
4	Formate dehydrogenase
	Non-specific secondary alcohol dehydrogenase
	Non-specific aldehyde dehydrogenase

Fig. 1. Route of oxidation of methane by methanotrophs.

The lack of specificity of these enzymes is expressed in vivo in some methanotrophs, which, on incubation with a wide range of hydrophobic compounds, in the presence or absence of methane, yield one or more partial oxidation products. To date only a few species have been tested for biotransformation activities toward a wide range of compounds and the published range has doubtless been restricted by commercial considerations, which have led to the filing of several patents (15). Intact organisms catalyze not only oxidations, but also dehalogenations, substituent shift reactions in aromatic compounds and alkyl condensation reactions (15,19). The mechanistic basis for these is not understood.

These biotransformations have been described as fortuitous metabolism (51,53). In some cases, however, there is evidence that products arising from the initial oxidation of substances commonly found in the environment (e.g., fatty acids formed from _n_-alkanes) are degraded and carbon and energy for growth may be derived from

these heterotrophic processes, in which case the term 'supplementary metabolism' might be more appropriate (15).

There is clearly potential for exploiting these catalytic activities, either using isolated enzymes or intact organisms. The obligate methanotrophs offer some special advantages for development of whole organism transformation processes compared to heterotrophs and these are discussed below. Unfortunately, at present these inherent advantages may be partly undermined by difficulties experienced in developing genetic systems for these bacteria. On the other hand, the recently isolated facultative methanotrophs, (31,42) whilst lacking some of the advantages of the obligate strains, seem to be more amenable to conventional genetic techniques. Their biotransformation activities have not yet, however, been examined in any detail.

In addition to catalyzing chemical transformations, methanotrophs may prove useful as sources of biopolymers. For example, under some growth conditions, a substantial proportion of the biomass of some species is poly-β-hydroxybutyrate (PHB) (2,12,27).

Since many C_1 genes are apparently not repressible in obligate methanotrophs, use of their operons as promotors for expression of cloned eukaryotic genes may be valuable for production of eukaryotic proteins. For recent, detailed reviews of this group of microorganisms, see references 6, 14, 16, 60 and 64.

ENZYMES RESPONSIBLE FOR BIOTRANSFORMATION ACTIVITIES OF METHANOTROPHS

The most important enzymes responsible for these biotransformation processes are shown in Figure 1. Other enzymes involved include those that generate reducing power (required by MMO) e.g., enzymes which mobilize PHB or β-oxidise fatty acids.

Methane Monooxygenase (MMO)

Although such activity (usually requiring NAD(P)H) has been reported in cell-free extracts of a range of methanotrophs over the last 11 years, these enzymes are notoriously unstable and work in several laboratories has been plagued by lack of reproducibility. In most cases the activity is found in particulate fractions (64) from disrupted organisms but in some species only soluble activity has been described (4). At least in some species, the location of MMO depends upon growth conditions (17,48,52).

MMO has been purified from two dissimilar species, *Methylococcus capsulatus* Bath (4) and *Methylosinus trichosporium* OB3b (54) but recently stability problems have prevented purification of the enzyme

from the latter organism. Although in both cases a three component system was isolated, they were not identical. Both soluble and particulate activities are found in the latter species depending upon growth conditions. Whilst the soluble activity appears to be similar to that in Mtlc. capsulatus Bath (52) the particulate activity is different in some respects, most notably its sensitivity to a range of inhibitors (17,48).

Currently, the best characterized MMO appears to be the Mtlc. capsulatus (Bath) system (8,52). One component (A) is oxygen labile and is thought to be the hydroxylase which, in the reduced form, binds the substrate (8). It has a molecular weight of 220,000 with 2 non-haem iron atoms and 2 acid-labile sulphur atoms. Component B, a colorless protein of molecular weight about 15,000, is essential for oxygenase activity, although its role is uncertain. Component C, the reductase, with a molecular weight of 44,000 contains 1 molecule of FAD, 2 atoms of non-haem iron and 2 atoms of acid-labile sulphur and is reduced by NADH. The particulate Mts. trichosporium OB3b system comprised two membrane bound components (molecular weights, 47,000 and 9,400 respectively) and a CO-binding cytochrome-c (molecular weight 13,000) found mainly in the soluble fraction of disrupted organisms (54).

The soluble enzyme from Mts. trichosporium OB3b cell-free extracts can be resolved into two components by ion-exchange chromatography (52). Reconstitution of the two fractions resulted in reduced activity but full activity could be restored to Fraction 1 by addition of purified components B and C from Mtlc. capsulatus (Bath) suggesting some homogeneity between these two soluble enzymes (52).

In most cases, NAD(P)H appears to be the only suitable electron donor for MMO (4,5,10,17,40,46,48,49,52); this may present problems of supply of this cofactor to the MMO during exploitation of this enzyme as a biocatalyst using both in vitro and in vivo systems. Ascorbate mediated, in vitro MMO activity has been reported for two organisms (43,54,55), which may indicate the involvement of a CO-binding cytochrome c in the transfer of reducing equivalents from methanol dehydrogenase to MMO (55). Although this would not involve the use of reduced pyridine nucleotides, such a system may only be present under certain growth conditions (3,17,48). Cell-free MMO from Mts. trichosporium OB3b and Mtlc. capsulatus (Bath) can oxygenate a diverse range of substrates, although the enzyme system from Methylomonas methanica is more specific (7,49). Broad substrate MMO activity is expressed in vivo (8,15,17,19) but some products are subject to further oxidation by other non-specific oxido-reductases. In addition, some unusual activities, e.g., dehalogenation, may be due to MMO or manifestions of other enzymes. Examples of cell-free and whole organism catalytic activities for one species are shown in Table I.

TABLE I
Examples of Biotransformations Effected by
Methylosinus trichosporium OB3b

Cell-free oxidations

Compounds	Products	
CH_4 Methane	CH_3OH Methanol	HCHO Formaldehyde
$CH_3(CH_2)_2CH_3$ Butane	$CH_3(CH_2)_3CH_2OH$ Butan-1-ol	
$CH_2 = CH_2$ Ethene	$CH_2 - CH_2$ (bridged by O) Ethene oxide	
$CH_3.CH = CH_2$ Propene	$CH_3.CH - CH_2$ (bridged by O) Propene oxide	
$C_6H_5CH = CH_2$ Styrene	$C_6H_5CH - CH_2$ (bridged by O) Styrene oxide	
$C_6H_5C_2H_5$ Ethylbenzene	$HO-C_6H_4-C_2H_5$ *p*-Hydroxyethylbenzene	

Whole organism transformations

Type of reaction	Compound	Products
Oxidation	Ethene	Ethene oxide
	Propene	Propene oxide
	$CH_2 = CH - CH = CH_2$ But-1, 4-diene	$CH_2 - CH - CH = CH_2$. (with O bridging the first two carbons) 1,2 Epoxy-but-4-ene
	Benzene	OH Phenol
	CH_3 CH CH_3 CH_3 p-Cymene	COOH CH CH_3 CH_3 p-Cumic acid CH_3 CH CH_3 CH_2OH 2-Tolyl-propan-1-ol
Oxidation and Degradation	$CH_3(CH_2)_{14}CH_3$ Hexadecane	$CH_3(CH_2)_{14}CH_2OH$ Hexadecan-1-ol CO_2
	C_7H_{15} 1-Phenylheptane	OH $CH(CH_2)_5CH_3$ 1-Hydroxy-1-phenyl-heptane CH=CH.COOH Cinnamic Acid COOH Benzoic Acid
Oxidation and Dechlorination	CH_3 Cl m-Chlorotoluene	CH_2OH Benzyl alcohol H_2C O * Benzyl epoxide

* tentative identification

Type of reaction	Compound	Product
Oxidation Dechlorination and Condensation	m-Chlorotoluene	m- or p-Methyl benzyl alcohol
Oxidation and Condensation	m-Chlorophenol	An hydroxyphenylheptanoic acid *
Oxidation and Rearrangement	m-Cresol	m- and p-Hydroxybenzaldehyde
	o-Cresol	5-Methyl-1,3-benzene diol

* tentative identification

Methanol Dehydrogenase

The second enzyme of the C_1 oxidative pathway, methanol dehydrogenase has been purified and characterized from several methane-oxidizing bacteria and all the activities appear to be closely similar (36-38,58). The cell-free enzyme can be coupled to phenazine - (m) ethosulphate and usually requires ammonia or a primary amine as an activator. Methanol dehydrogenase has a wide substrate range but is generally specific for primary alcohols, although some secondary and tertiary alcohols are slowly oxidized by the enzyme from Methylbacterium organophilum strain XX (63). The prosthetic group of methanol dehydrogenase has now been fully characterized as a pyrolloquinoline quinone (Methoxatin) (9,47). We are developing electrochemical procedures for the oxidation and reduction of this enzyme which may facilitate its use with or without MMO in biocatalytic systems (18) and it has been used as the basis of an enzyme fuel cell and sensing device (44).

Formaldehyde Dehydrogenase

Although formaldehyde is a substrate for methanol dehydrogenase by virtue of its hydration configuration, methylotrophs possess several types of formaldehyde dehydrogenase activity encompassing enzymes which are specific for formaldehyde and non-specific aldehyde dehydrogenases. A $NAD(P)^+$-linked formaldehyde dehydrogenase has been purified from Mtlc. capsulatus (Bath) (50) and preliminary evidence suggests a NAD^+-linked enzyme is present in Mts. trichosporium OB3b (52). $NAD(P)^+$-independent activities, requiring phenazine methosulphate have been demonstrated in extracts of several methane-utilizing organisms (39) and a heme-containing non-specific aldehyde dehydrogenase of this type has been purified from Mts. trichosporium strain PG.

Other Oxidoreductase Enzymes

Formate is oxidized to carbon dioxide by a NAD^+-linked formate dehydrogenase present in several methanotrophs (64). Little is known of this activity toward other substrates, but it is important in supplying NADH to the MMO during co-oxidations. The secondary alcohol dehydrogenase that has been reported in several methane- and methanol-utilizers is also NAD^+-dependent (24,25). Whether this enzyme would be important in either further oxidations or supplying reducing power is uncertain in view of its relatively limited substrate range (24).

EXPLOITATION OF CELL-FREE SYSTEMS FOR CHEMICAL TRANSFORMATION

In view of the complexity of the methane monooxygenase enzyme described in various methanotrophs, *in vitro* exploitation of the purified enzyme system poses many problems. Its notorious instability has pre-empted, to date, any attempts to immobilize the system and it may prove extremely difficult to assemble the multicomponent complex in an active immobilized form. Should this be achieved, however, a further problem is the supply of reducing power to the immobilized complex. One plausible approach to this would involve the co-immobilization of formate dehydrogenase to regenerate NADH during the oxidation of formate to carbon dioxide and water. An alternative approach would be the chemical reduction of the cofactor or direct electrochemical reduction of an MMO component (20,21). There is little detailed knowledge of the response of such a system to either the types of organic substrates presented for oxidation or the nature of interaction of a three phase system (gaseous-hydrophobic-hydrophilic) at an immobilized enzyme surface. Thus, despite the apparent attraction of a single immobilized MMO enzyme system for specific oxidation of a range of organic substrates, without the concomitant problems of further metabolism effected by other non-specific oxidoreductases, such a system is not yet possible due to enzyme instability. Perhaps an ideal system would comprise a simple enzyme electrode in which the hydroxylase component alone is immobilized and is reduced electrochemically. In the short term whole organism transformation systems offer a more feasible alternative.

EXPLOITATION OF WHOLE ORGANISM BIOTRANSFORMATION SYSTEMS

The exploitation of intact methanotrophs to catalyze specific oxidations could involve use of either immobilized or non-immobilized organisms. It has been found that the nature of the matrix used during the immobilization of methane-oxidizing organisms critically affects their oxidative capacity (28). The MMO activity of immobilized organisms was significantly lowered but immobilized *Methylomonas rubra* oxidized methane for up to nine days without any further loss of catalytic activity. Immobilized systems may therefore be less suitable for this type of catalytic activity than whole organism suspensions.

The range of oxidations expressed by such resting suspensions is species-dependent (19,51), (although a comprehensive survey of methanotrophs with regard to their oxidative capacities has not been made). This may reflect differences in the nature of MMO itself or in the rate and extent of entry of potential substrates into the organism. One of the most notable features of the broad specificity

oxidations effected by methanotrophs is the extracellular accumulation of oxidation products; this is especially true for the class of oxidation products, such as epoxides, that are not further oxidized. If the oxidation products are further metabolized then their accumulation is dependent upon the relevant K_m of subsequent oxidoreductases. For instance, during the oxidation of ethane or toluene by whole organism suspensions of Mts. trichosporium OB3b, neither of the corresponding alcohols accumulates, but there is transient build-up of acetaldehyde and benzaldehyde respectively, before their further oxidation to the corresponding acids. Similarly, secondary alcohols accumulate transiently before oxidation to the corresponding methyl ketones (26,41).

The rate of oxidation exhibited by MMO in whole organisms is dependent not only upon species but also upon the nutritional status of the organism, a situation which reflects the nature of the supply of reducing power to the MMO (8,16). The rate and extent of epoxide accumulation (Table I) is species dependent (8,22). We have found that the rate of epoxidation of propene to propene oxide by whole suspensions of Mts. trichosporium OB3b is affected by the nutritional status of the culture. Freshly harvested organisms from continuous culture under methane excess conditions, containing up to 30% of the dry weight as the storage polymer, PHB, possess high endogenous propene epoxidation activity (30-60 nmoles. min^{-1}. mg. dry wt^{-1}). In contrast, those derived from carbon-limited cultures or starved at 30°C for 24-48 hours possess low activities (0-15 nmoles min^{-1}. mg.dry wt^{-1}). Restoration of epoxidation rates can be achieved by addition of an exogenous source of reducing power, such as methane metabolites (methanol, formaldehyde, formate) (23) or other substrates whose oxidation provides reducing power e.g., ethanol. Thus, in assessing the comparative capabilities of strains to perform biotransformations the physiological state is of prime importance. These findings illustrate that the provision of reducing power to the MMO in whole organisms is easily achieved.

There is little information concerning the mode of entry of many of the substrates into these microorganisms, whether it occurs by simple diffusion or active transport. Similarly, little is known of the effect of many of these hydrophobic substrates upon the structure of the organisms. They may act as permeabilizers or metabolic inhibitors. Methanol, the primary oxidation product of methane, acts both as a substrate and an inhibitor for MMO and it is possible that the primary hydroxylated products of certain hydrophobic substrates may cause inhibition. Studies in our laboratory have shown that there is a drastic reduction in the activity of MMO concomitant with the accumulation of propene oxide, during the oxidation of propene by whole organism suspensions of Mts. trichosporium OB3b (Figure 2). Similarly, pre-incubation of suspensions with low concentrations of propene oxide (1mM) for 1 hour showed similar drastic loss of epoxidizing activity. However, addition of formate restored the oxidizing activity to the original levels (Fig. 3) show-

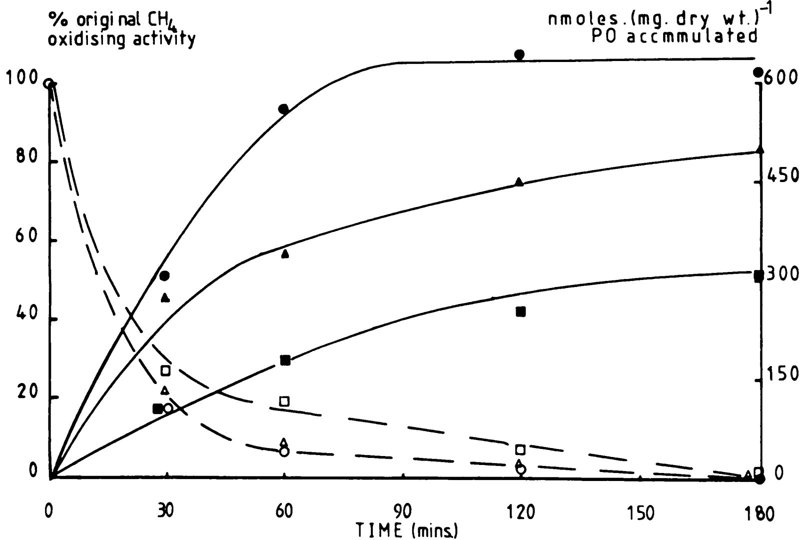

Figure 2. Effect of accumulation of propene oxide from propene on the methane oxidizing capacity of a suspension of Methylosinus trichosporium OB3b. Suspensions of the methanotroph (10 mg dry wt/ml in 20 mM sodium phosphate buffer pH 7.0) were incubated in sealed conical flasks (50 ml) containing air-propene (■ 1 ml: ▲ 2 ml: ● 5 ml) mixtures. The flasks were shaken at 30°C in a reciprocating water bath (100 cycles/min). Samples (50 µl) were withdrawn periodically for polarographic determination of methane-oxidizing activity. Propene oxide was determined gas chromatographically.

ing that inhibition was not due to end product inhibition of MMO but to inhibition of the supply of reducing power to MMO, in this case via the mobilization of intracellular PHB reserves. The actual degree to which the primary oxidation products inhibit the system may well depend upon the degree to which accumulation of product is extracellular.

In exploiting the broad oxidative capacity of methanotrophs, a further drawback is one characteristic to heterotrophs, namely that for many substrates the primary oxidation products are further metabolized: primary alcohols may be oxidized to aldehydes, aliphatic acids and subsequently β-oxidized (15); secondary alcohols that accumulate initially may be oxidized to ketones via the action of secondary alcohol dehydrogenase; some oxidation products may be further oxidized by methane monooxygenase itself. For example, ethyl benzene is converted under certain circumstances of substrate limitation, first to a mono- and subsequently to a di-hydroxy derivative. In the latter case the extent of hydroxylation initiated by MMO may be determined by the substrate concentration.

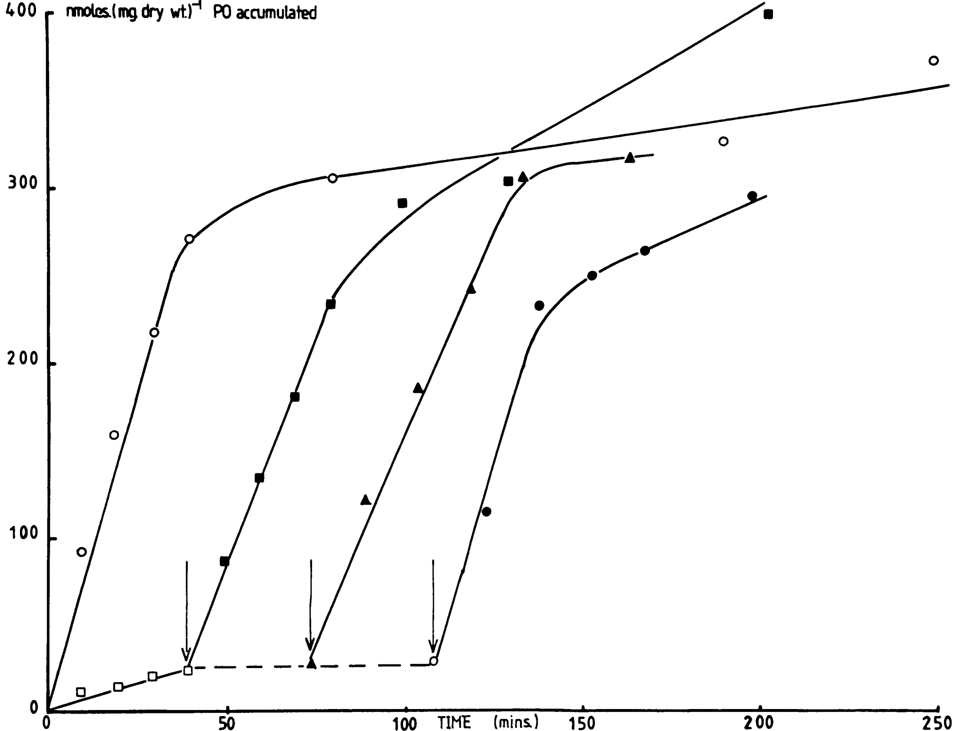

Figure 3. Restoration of biotransformation activity of a propene oxide-inhibited suspension of Methylosinus trichosporium OB3b by addition of formate. Suspensions of the methanotroph (10 mg dry wt/ ml in 20 mM sodium phosphate buffer, pH 7.0) were incubated with air propene (5 ml) mixtures in sealed conical flasks (50 ml). O, endogenous activity; □, activity after preincubation for 1 hr with propene oxide (1 mM): ■, ▲, ●, effect of adding sodium formate, 50, 25 and 10 mM respectively.

Some of the problems in developing chemical transformation systems based on methanotrophs and discussed above may be resolved by genetic techniques.

POTENTIAL FOR GENETIC MANIPULATION OF METHANOTROPHS FOR INDUSTRY

Methane and methanol are likely to remain relatively inexpensive substrates, in the foreseeable future, for microbial growth. There is already substantial industrial experience of cultivation of methylotrophs and commitment to methylotrophic SCP (single cell protein) processes. The long term industrial future for methanotrophs in particular may well be in part dependent on the development of genetic systems for these microorganisms. Given our currest state of knowledge and excluding genetical considerations, the obligate

species show more promise than the facultative ones since the latter are generally slower growing and less stable (11). There are currently only a few facultative isolates, however, and more suitable strains might be forthcoming.

Although obligate methanotrophs have been studied since the beginning of this century, paradoxically we know less about their genetics than those of facultative species first described only a few years ago. This is because of difficulties experienced in obtaining phenotypic mutants. On treatment with N-methyl-N-nitro-N-nitrosoquanidine (MNNG) such mutants arise at a frequency of about 10^{-3} but the reversion frequency is very high (13,62). Mutants were not obtained using other chemical mutagens, UV or γ irradiation. In contrast, both MNNG and UV mutagenesis yield auxotrophic mutants of facultative methanotrophs which can readily be transformed to prototrophy by exogenous wild-type DNA (35).

It seems unlikely that inability to isolate mutants of obligate methanotrophs is due to fundamental differences in their genetic systems but perhaps inappropriate techniques have been used. For example, at least some methanotrophs are probably not mutable by UV due to lack of error-prone 'SOS' DNA repair mechanisms.

Lytic bacteriophages for methanotrophs have been detected (56, 57) and in facultative species the MMO may be plasmid encoded (65). In the facultative methanotroph, Methylobacterium organophilum, O'Connor and Hanson (32-34) have shown by DNA transformation experiments that many of the key enzymes of C_1 oxidation and assimilation are co-ordinately regulated and appear to be located on a single operon.

In view of their rather special 'lifestyle', obligate methanotrophs might be expected to have some rather unusual metabolic regulation mechanisms. Many of their oxidative enzymes are probably constitutive and not subject to catabolite repression. For example, in our hands the MMO of Mts. trichosporium is present after growth of methanol with similar activity to that during growth on methane (2). Interestingly, growth of this species on methanol requires a long period of adaptation (2). Although they are not required tc show the metabolic versatility of many heterotrophs, obligate methanotrophs clearly have sophisticated genetic regulation systems as exemplified by their ability to undergo encystment (43,61) and to regulate their intracellular morphology (16) and MMO type and localization (16) in relation to growth conditions. Major variations in membrane proteins have been observed under different growth conditions (Figure 4).

In order to understand metabolic regulation in methanotrophs and to have the capacity to develop strains for particular industrial purposes, there is a pressing need to develop genetic systems.

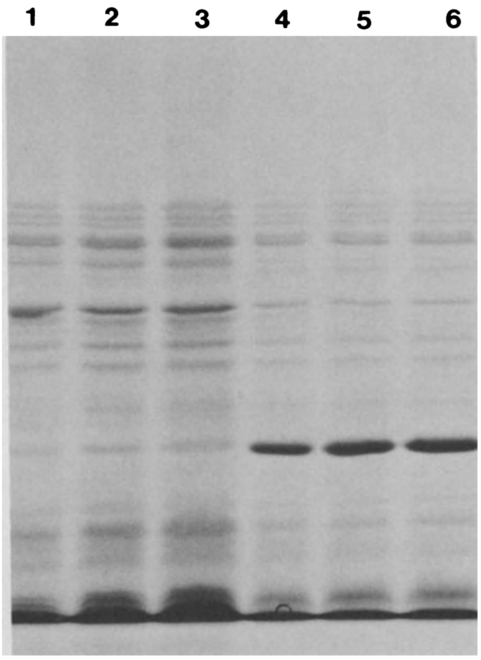

Figure 4. SDS PAGE analysis of total membrane proteins of Methylosinus trichosporium OB3b, grown under different conditions. Tracks 1-3: Membrane proteins during oxygen-limited chemostat culture (10, 15, 20 μg protein loaded respectively). Tracks 4-6: Membrane proteins during oxygen-limited growth in shake flasks (10,15,20 μg protein loaded respectively).

The use of promiscuous plasmids both to generate mutants by transposon mutagenesis and map mutants by chromosome mobilization offers the best route to these ends. In this regard, we have already demonstrated transfer of the plasmid R68.45 into Mts. trichosporium and expression of antibiotic resistance markers therein (59).

Until a reasonable understanding of methanotrophic genetics is established, it is difficult to predict the industrial potential of genetically modified strains. Obvious possibilities, however, include engineered strains with increased MMO content or which synthesize valuable foreign proteins.

Multicopy plasmids could be used to increase MMO levels although this is not a simple project. The enzymes account for several percent of the total protein in wild-type bacteria, are multi-component containing metal ions and prosthetic groups, and, at least in some species, vary in type with different growth conditions. MMO elaboration is, therefore, likely to be a complex procedure involving a

number of genes. An order of magnitude increase in MMO content may be theoretically possible but it would be surprising if it could be achieved in practice; a 3- or 4- fold increase would be a more realistic aim. Even then, it is unlikely that the increased enzyme content would be expressed in vivo due to several factors, most notably limitations on the supply of reducing power. Achieving an increase in this also would demand some very elaborate genetic engineering. Nevertheless, this would be a valuable approach for increasing the efficiency of production of MMO for in vitro use. Such exploitation, however, awaits development of procedures for stabilization of the enzyme.

As discussed above, there may be advantages in using methanotrophs as vehicles for expression of foreign DNA and this type of strain construction could usefully be done prior to establishing a detailed knowledge of methanotrophic genetic systems.

CONCLUSION

It should be evident from the preceeding discussion that methanotrophs offer considerable opportunity for industrial exploitation, not only for product synthesis but also for waste treatment. The first processes to be devised will most likely involve generation of high added value products, but as methanotrophic biochemical and genetical systems become better understood, a wider range of products and applications will follow.

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DISCUSSION

Q. OCONE: You spoke of the need for a co-reducing agent when the substrate of interest is not to be completely oxidized. Would molecular hydrogen serve?

A. HIGGINS: It is possible, but we have not yet tested hydrogen.

Q. RIBBONS: Is there a difference in substrate specificity of MMO in cells with and without membranes?

A. HIGGINS: On balance the evidence to date suggests there is no difference, but we need to test more substrates to answer this definitively.

THE ROLE OF NICKEL IN METHANOGENIC BACTERIA

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The methanogenic bacteria are strict anaerobes which synthesize methane from H_2 and CO_2, acetate, formate, methanol, or methylamines. They have two particularly relevant applications: in the anaerobic decomposition of waste materials and the conversion of biomass to fuel. Because of the economic importance of these processes, methanogens might be considered likely candidates for genetic engineering. However, two problems arise. First, methanogenic bacteria are members of the archaebacteria (28). From what is known about these organisms, we can expect that their biology will be as different from the eubacteria as the biology of the eubacteria is different from the eucaryotes. This point was elaborated by John Reeves in this volume. Secondly, not much is known about the biochemistry of the methanogenic bacteria. This is best illustrated by a short description of our knowledge concerning methane synthesis from H_2 and CO_2 (Fig. 1). Here we consider methanogenesis as occurring by the stepwise reduction of CO_2 to methane. Of the five steps described here, only reaction V, the methylreductase reaction, has been described in detail. As we will show, a great deal more needs to be known about the methylreductase. So for the present time genetic engineers will have to cope with an unfamiliar organism and a largely unknown biochemistry.

Recently our attempts to elucidate the mechanism of methane synthesis have led us to consider the role of nickel in methanogenic bacteria. Nickel is an element of low natural abundance which has proven of only occasional interest in biology. Nickel is present

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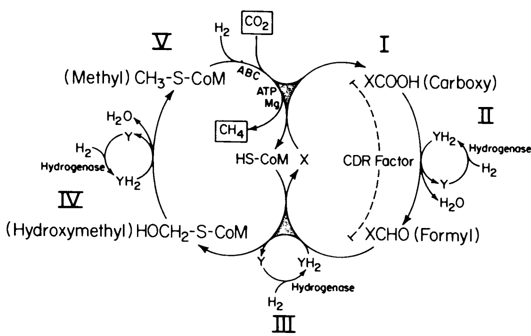

Figure 1. Proposed model for the reduction of carbon dioxide to methane (28) modified from Romesser (19). The carrier(s) at reactions I, II, and III are unknown. The nature of "Y" is also uncertain. CDR (carbon dioxide reducing) factor is a low molecular weight compound of unknown structure required for the reduction of carbon dioxide in cell extracts. Reaction V is examined in greater detail below. Reproduced with permission from Antonie van Leeuwenhoek (28).

in only 80 ppm in the earth's crust and at concentrations one hundred thousand times lower in sea water (25). These concentrations are comparable to those of zinc and cobalt. In contrast, iron is about one thousand times more abundant than nickel. Nevertheless, nickel has been found to be essential for the growth of some plants and microorganisms under certain conditions (24). In particular, nickel is required for autotrophic growth (1) and synthesis of an active hydrogenase (11) in the Knallgas bacteria. Acetogenic bacteria and some clostridia required nickel for the synthesis of an active carbon monoxide dehydrogenase (4, 7). In addition, nickel appears to be essential for the function of urease from jack beans (6) and rumen bacteria (22).

The importance of nickel in methanogenic bacteria was first appreciated in the laboratory of R. Thauer (21). While trying to determine the cause of erratic growth yields of *Methanobacterium thermoautotrophicum*, they noticed that high growth yields were only obtained when a stainless steel probe was used in the otherwise all-glass fermentor. The addition of nickel chloride to the medium substituted for the stainless steel probe. Subsequently, the growth yields were shown to be dependent on the concentration of nickel in the medium (Fig. 2A). At low concentrations of nickel, 150 nmol Ni^{2+} is required for the formation of 1 g dry weight of cells. Presumably, the nickel requirement had previously been overlooked because Ni^{2+} is also a contaminant in other components of the media.

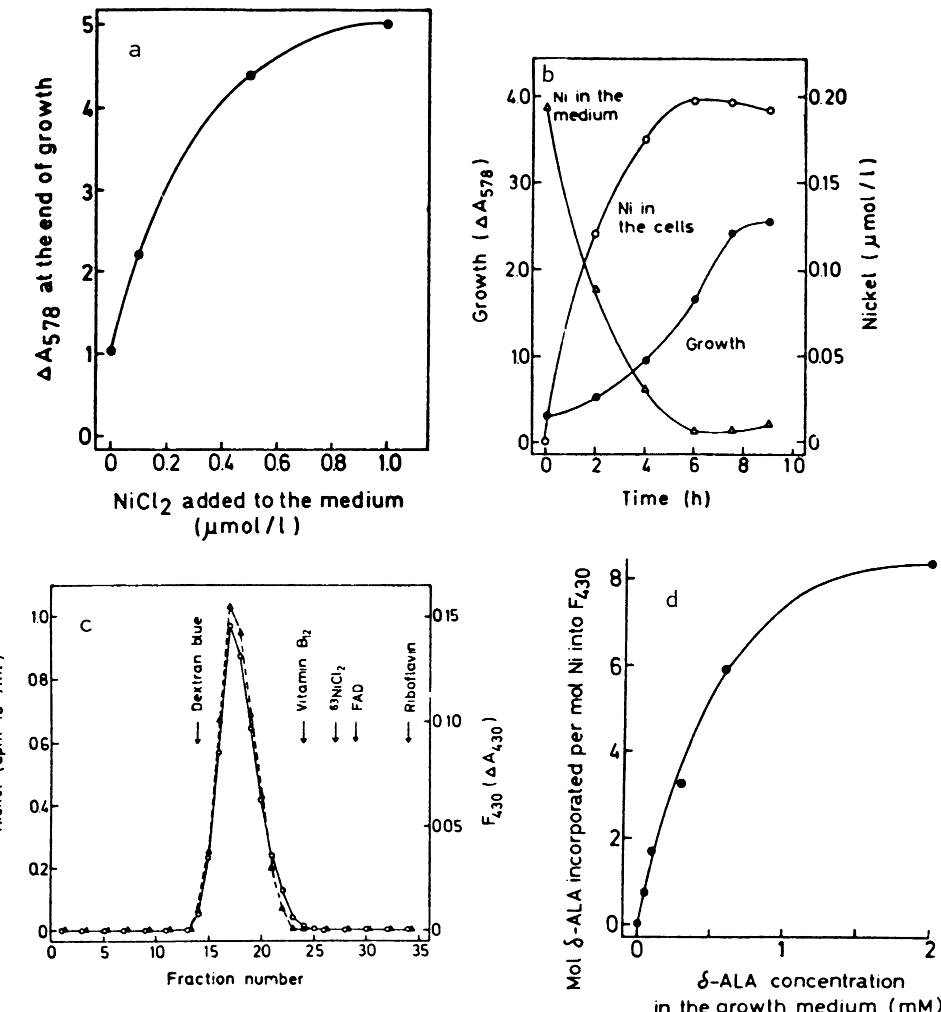

Figure 2. A. Nickel requirement for the growth of M. thermoautotrophicum. Reproduced with permission from Archives of Microbiology (21). B. ^{63}Nickel uptake by M. thermoautotrophicum. Reproduced with permission from Archives of Microbiology (3). C. ^{63}Nickel (O) and F-430 (▲) elution profile from Sephadex G-25 column (1.2 x 50 cm). Arrows indicate peak fractions of reference substances. Reproduced with permission from Archives of Microbiology (3). D. Mol δ-aminolevulinic acid incorporated/mol nickel into F-430 in relation to the concentration of δ-aminolevulinic acid in the growth medium. Reproduced with permission from FEBS Letters (2).

Further studies by Thauer's group (3) showed that the radioactive isotope of nickel, ^{63}Ni, was quantitatively taken up by whole cells (Fig. 2B). In cell-free extracts of labeled cells, most of this radiolabel was associated with the high molecular weight components of the cell. However, after heat treatment 90% of the label was found to be soluble in 70% aqueous acetone. Chromatography of the acetone soluble fraction showed that the radiolabel comigrated on ion exchange and molecular sieve columns with a yellow compound known as Factor 430 (Fig. 2C). Furthermore, the amount of F-430 that could be isolated from whole cells of *M. thermoautotrophicum* (5) depended on the amount of Ni^{2+} in the growth medium (Table I). Therefore, they concluded that nickel was a component of F-430.

TABLE I
F-430 Content of *M. thermoautotrophicum* Grown with different concentrations of $NiCl_2$[a]

	I	II	III
Ni in medium (μM)	0.075	0.32	2.50
Cell yield (g dry wt/L)	0.68	1.12	1.80
Ni uptake (nmol/g cells)	110	275	1160
F-430 content (nmol/g cells)	25	135	695

[a]Adapted from G. Diekert *et al.* (5).

Factor 430, named because of a visible absorption maximum at 430 nm, had been discovered by Jean LeGall in extracts of *M. thermoautotrophicum* several years earlier. R. Gunsalus partially purified the compound and found that it was non-fluorescent and neither easily oxidized nor reduced (12). The molecular weight was estimated to be greater than 1000. Because of these unusual properties, quantities sufficient for neutron activation analysis were purified from *Methanobacterium bryantii* (26). These studies were performed concurrently with the radiolabel-tracer-studies in Thauer's laboratory. The results indicated that stoichiometric amounts of nickel were bound to F-430 and that no other metals were present in significant amounts (Table II). Thus, two completely independent approaches led to the same conclusion. To our

knowledge, no similar low-molecular-weight nickel-compounds have previously been isolated from biological material. Thus, there is an obvious interest in the structure and function of this novel compound.

TABLE II
Neutron Activation Analysis of two Forms of Factor 430.[a]

Metal	PPM F-430a	PPM F-430b
Co	23.0	104.0
Cu	840.0 ± 120	<320.0
Fe	<0.5	<0.8
Mn	17.3 ± 0.4	49.4 ± 0.7
Mo	<37.0	<58.0
Ni	17 600.0 ± 830	39 400.0 ± 1800
zn	400.0 ± 80	460.0 ± 120

[a]Data given ± standard deviation. From reference 26.

At this point, little is known about the structure of F-430. When (^{14}C)δaminolevulinic acid is added to the medium for M. thermoautotrophicum, the radiolabel is incorporated into F-430 (2). As the amount of δ-ALA is increased, the amount of radiolabel incorporated into F-430 reaches a maximum corresponding to eight molecules of δ-ALA per atom of nickel (Fig. 2D). Based on this evidence, F-430 may well be a tetrapyrrole. The complete structure of F-430 is still an area of active research.

Recent experiments in our laboratory may illuminate the function of F-430. We have been studying the biochemistry of methane synthesis in cell free extracts of M. thermoautotrophicum (23, 29). The terminal step in this pathway (Fig. 3) is the reduction of 2-(methylthio)ethanesulfonic acid (CH_3-S-CoM) to form methane and 2-mercaptoethanesulfonic acid (HS-CoM). In addition to an electron donor, this reaction required Mg^{2+} and catalytic amounts of ATP (13, 18). Fractionation of cell-free extracts (14) separates three components necessary for the activity when H_2 is the electron donor (Fig. 4). Component A is a large protein complex that contains

$$\underset{CH_3\text{-}F\text{-}CoM}{CH_3SCH_2CH_2SO_3^-} + H_2 \xrightarrow[A,\ B,\ C]{ATP,\ Mg^{2+}} CH_4 + \underset{HS\text{-}CoM}{HSCH_2CH_2SO_3^-}$$

Figure 3. Reaction catalyzed by the methylreductase system.

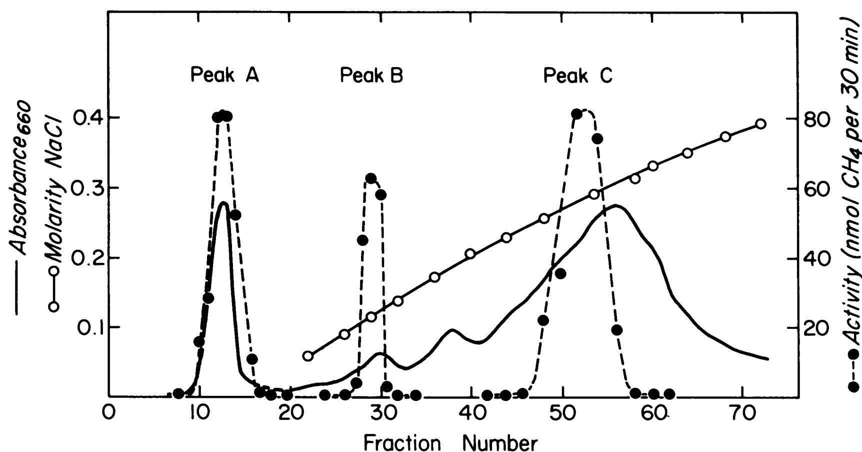

Figure 4. Resolution of the methylreductase system for components A, B, and C by DEAE-cellulose ion exchange chromatography. Reproduced with permission from the Journal of Biological Chemistry (14).

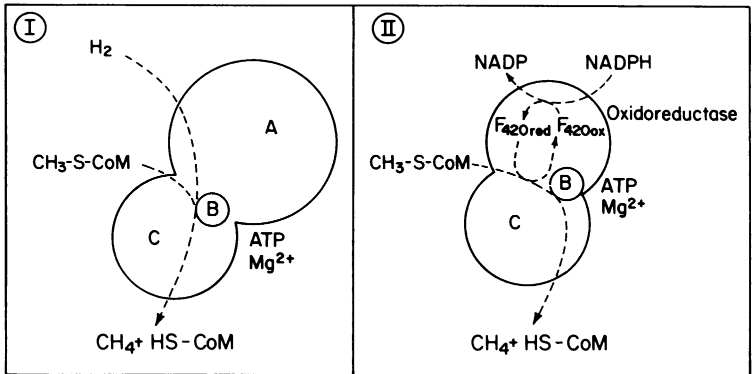

Figure 5. Diagram of methylreductase system to show possible relationships, when (I) electrons are supplied via component A hydrogenase or (II) when electrons are supplied by an NADPH-F-420 oxidoreductase. Reproduced with permission from Ellefson and Wolfe (10).

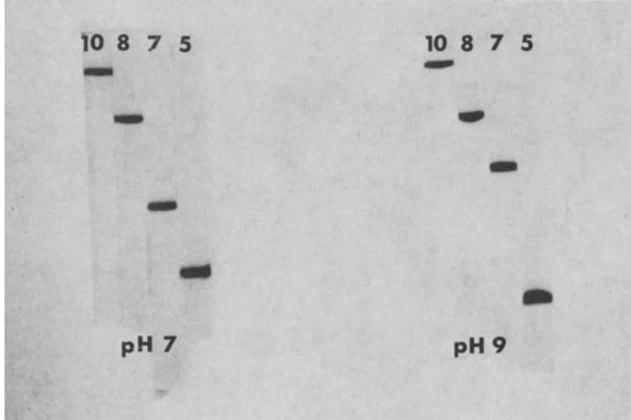

Figure 6. Polyacrylamide gel electrophoresis of purified methylreductase. Electrophoresis was performed at pH 7 and pH 9 and the following polyacrylamide concentrations: 10%, 8%, 7%, 5%. Reproduced with permission from Journal of Biological Chemistry (9).

hydrogenase as well as other proteins. Component B is a low molecular weight coenzyme. Component C is a protein complex. However, if NADPH is substituted for H_2, component A can be eliminated from the reaction (8). In addition to components B and C, this reaction requires (8) an unusual deazaflavin coenzyme (F-420), ATP, and Mg^{2+} (Fig. 5). Therefore, component C must contain the methylreductase and presumably other enzymes including a NADPH-F-420 oxidoreductase.

The methylreductase has now been purified to homogeneity (9) from component C of M. thermoautotrophicum (Fig. 6). The native protein has a molecular weight of 300,000 and is composed of three different subunits with molecular weights of 68,000, 45,000, and 38,500. The subunits are present in equal proportions, suggesting a stoichiometry of α_2, β_2, γ_2 in the native protein. In addition, antibodies specific to the methylreductase have been raised in the mouse. This antiserum crossreacts with proteins in cell-free extracts from other methanogenic bacteria. These crossreacting proteins comigrate on polyacrylamide gels (Fig. 7) with the methylreductase from M. thermoautotrophicum as demonstrated by immunoreplicate electrophoresis (9). Therefore, this enzyme appears to be widely distributed among methanogenic bacteria. This result is consistent with the idea that the methylreductase is a major component of the methane synthesizing system in vivo.

The homogeneous methylreductase also has an intense yellow color (9). In the visible spectrum, there is an absorption maximum at 425 nm and a shoulder at 445 nm (Fig. 8). This chromophore is non-fluorescent. Furthermore, no changes in the visible spectrum are observed subsequent to treatment of the enzyme with the reduc-

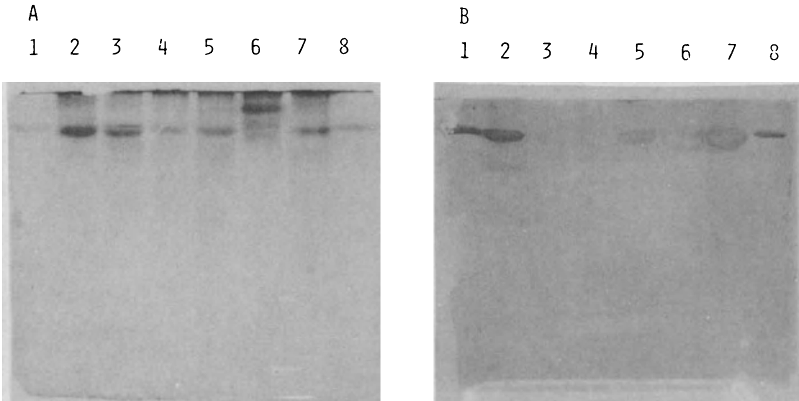

Figure 7. Immunoreplicate electrophoresis of the methylreductase and cell extracts of other methanogens. The methylreductase from M. thermoautotrophicum and cell extracts from various methanogens were first subjected to polyacrylamide gel electrophoresis (A) and then overlaid on an agarose gel containing antibodies to purified methylreductase to obtain the immunoreplicate (B). Lanes 1 and 8 contained 2 μg purified methylreductase; other lanes contained cell extract as indicated: lane 2, M. thermoautotrophicum (25 μg); lane 3, Methanogenium marisnigri (25 μg); lane 4, Methanobrevibacter ruminantium (25 μg); lane 5, Methanobacterium formicicum (25 μg): lane 6, Methanospirillum hungatei (25 μg), and lane 7, M. bryantii str. MOHG (25 μg). Reproduced with permission from Journal of Biological Chemistry (9).

tants ascorbate, dithionite, and sodium borohydride or the oxidant ferricyanide (9). However, when the enzyme is extracted with boiling 80% methanol, a yellow compound with an absorption maximum at 430 nm is separated from the protein. The UV-VIS spectrum of the purified chromophore is identical to the spectrum of F-430. This chromophore also shows very similar chromatographic properties on TLC and HPLC to F-430.

Several lines of evidence demonstrate that the chromophore contains nickel. When the methylreductase is purified from cells grown on medium containing ^{63}Ni, the radiolabel copurifies with the enzyme. The radiolabel also comigrates with the methylreductase during electrophoresis on polyacrylamide gels and is precipitated by antiserum specific to the methylreductase. Extraction of the enzyme with boiling 80% methanol separates the ^{63}Ni from the protein. Chromatography of the methanol-extracted material on TLC or HPLC demonstrates that the radiolabel has an identical mobility with the chromophore from the methylreductase. Thus, the chromophore of the methylreductase contains nickel and appears to be a form of F-430. Whether or not it is identical to one of the forms

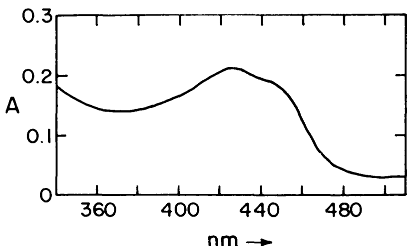

Figure 8. Visible adsorption spectrum of the methylreductase. Reproduced with permission from Journal of Biological Chemistry (9).

of F-430 purified from whole cells (26) or crude extracts (3) is not known. Because the chromophore can be obtained by relatively gentle methods from active enzyme, its structure is probably very similar to the structure of F-430 *in vivo*.

The amount of nickel and F-430 bound to the methylreductase has been estimated by several methods. Atomic absorption measurements and the content of ^{63}Ni from radiolabeled enzyme both indicate that two moles of nickel are present per mole of enzyme. In addition, the amount of F-430 released from the enzyme by methanol-extraction is about 1.5 moles per mole of enzyme. Therefore, the methylreductase probably contains two moles of F-430 per mole of enzyme. This stoichiometry is consistent with the proposed subunit structure of α_2, β_2, γ_2 (9). The presence of stoichiometric amounts of F-430 in the methylreductase suggests that it may play some role in catalysis. However, we have been unable to successfully separate F-430 from the protein under conditions where enzymatic activity can be reconstituted. Therefore, direct evidence concerning the role of F-430 is lacking.

In addition to the role of nickel in F-430 and the methylreductase, there is some evidence which indicates that nickel may have additional roles in methanogenic bacteria. Washed membranes of *M. bryantii* contain an EPR center that can most easily be assigned to a Ni(III) complex (17). Unlike F-430, the EPR center is reduced by dithionite. Further studies will be necessary to clarify the precise chemical nature of this complex.

In conclusion, the presence of nickel in methanogenic bacteria has been firmly established. Most of the metal is found in a low molecular weight compound known as Factor 430. Factor 430 in turn is firmly bound to the methylreductase, the enzyme which catalyzes the terminal step in methane synthesis. Biosynthetic evidence indicates that F-430 is a tetrapyrrole. It is noteworthy that methanogenic bacteria are also an abundant source of corrinoids. In fact, the concentration of corrinoids, 0.52-0.66 μmol/g dry wt (15, 20), is very close to the concentration of F-430, 0.38-0.70

μmol/g dry wt (5, 26). Remarkably, cytochromes have not been detected in M. thermoautotrophicum (Gottschalk, personal communication) or M. bryantii (17) although they have been found in Methanosarcina barkeri (16). Conceivably, F-430 may serve as a rather unorthodox substitute for cytochromes in the electron transport system of methanogenic bacteria. Further investigations will be necessary to determine whether or not this is so. Alternatively, F-430 may function as a methyl carrier, in the activation of CO_2, or in some completely unrecognized function of the methylreductase.

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MOTHER NATURE LIKES SOME HALOGENATED COMPOUNDS

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I note that the title of this Conference indicates GENETIC ENGINEERING OF MICROORGANISMS in large print and in small print, "for chemicals". In this paper, you will have to be satisfied with the "for Chemicals" part of that title because we have not yet arrived at the genetic engineering phase. We soon hope to enter into genetic engineering especially with respect to the production of one of our halogenating enzymes and I will refer briefly to that at the end of this manuscript. I am sure you have seen the television commercials where Mother Nature, accompanied by flashes of lightning and loud noises says "Mother Nature likes this" or "Mother Nature doesn't like that". It was with that thought in mind that I arrived at a title for this paper. Most laymen and many scientists think only of industrial pollution and harmful chemicals when discussing roles for halogenated organic molecules in nature. However, I hope to persuade you that Mother Nature does like some halogenated compounds and indeed, has reserved halogenated compounds for some very special functions and purposes.

In general, naturally occurring halometabolites have potent biological activity and are made in small amounts. The grandmother of all these compounds is, of course, thyroxine (Fig. 1). This iodinated hormone is maintained in the blood stream of mammals at concentrations of about 1 x 10^{-9} molar. Thyroxine concentrations a little bit over that or a little bit under that level produce very dramatic effects. Thyroxine is unusual in that it is one of the halogenated compounds in nature where the iodine atoms can be replaced by other functional groups and biological activity can be maintained. For example, the iodine atoms can be replaced by methyl groups with maintenance of biological activity. Under these

Figure 1. Thyroxine.

circumstances, it is surprising that iodinated tyrosine residues have persisted through terrestrial evolution. As you know, there are large land masses where there is not sufficient iodide ion in the soil to maintain normal thyroid function. Thus you would think that natural selection would have produced mutants that could synthesize and utilize noniodinated thyroxine analogues. However, this is clearly not the case.

The next few figures take us through various parts of the microbial kingdom by listing one or two halogenating compounds produced by representative organisms from various large groups and phyla. Again, most of these compounds have been shown to have potent biological activity. Many of these halometabolites are antibiotics. They were discovered not because someone was searching for halometabolites, rather the search was for the ability of one microorganism to produce a compound which would inhibit the growth of another organism. In several cases when the inhibiting compound was finally isolated and characterized it was discovered that it contained one or more halogen atoms. A good example is *pyoluterorin*, an antibiotic produced by a marine bacterium (1,2). *Pyoluterorin* is produced by a marine pseudomonad and contains two chlorine atoms on a pyrole ring (Fig. 2). I am sure you are all familiar with aureomycin (3), an antibiotic which contains a chlorine atom situated on a phenolic ring (Fig. 2). Chloroamphenicol is another example of a potent antimicrobial agent which is produced by a member of the actinomyces family (4). This antibiotic contains a dichloroacetic acid side chain (Fig. 2).

Fungi are without doubt the most active terrestiral organisms with respect to the utilization of halogen anions for the production of halogenated compounds. Raistrick, an organic chemist who was quite active in the natural product field in the 1930's and 40's, tested 125 different species of fungi for their ability to synthesize halogenated compounds (5). Raistrick found that 77 of the 125 species tested were able to utilize and incorporate chloride ion into organic compounds. Some fungi were quite dramatic in terms of halometabolite production. Five of the 77 fungi tested were able, essentially, to incorporate all of the chloride ion that was added to the culture medium. Grieseofolvin (6) is a good example from this group of organisms (Fig. 3). Figure 3 also lists some other fungal halometabolites. Geodin and erdin are produced in high concentration by *Aspergillus terrus* (7,8).

EUBACTERIA

ACTINOMYCES

PYOLUTEORIN

AUREOMYCIN

CHLORAMPHENICOL

Figure 2. Bacterial halometabolites.

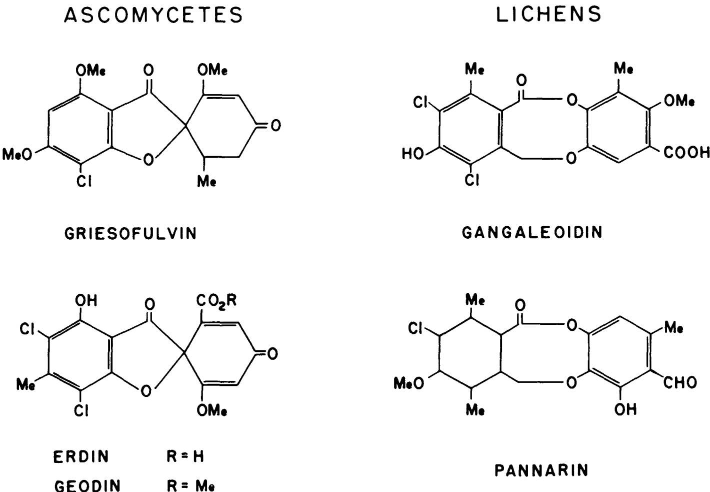

Figure 3. Fungal halometabolites.

13-chloro-1,14-docosane disulfate

13,16-dichloro-1,14-docosane disulfate

Figure 4. Chlorosulfolipids from Ochramonis danica. These compounds comprise 20% of the cellular lipids of O. danica (R. Vagelos).

Roy Vagelos and coworkers serendipitously discovered the chlorinated sulfolipids synthesized by a member of the protozoan family (Fig. 4). During studies on the comparative aspects of fatty acid biosynthesis, Vagelos and coworkers attempted to isolate the enzyme complex responsible for fatty acid biosynthesis in Ochramonis danica. Much to their dismay, they discovered that extracts from O. danica were always dead with respect to fatty acid biosynthesis. Not only were the O. danica extracts dead but they were also able to kill fatty acid biosynthesis in otherwise active extracts (9). The Vagelos group eventually traced this killing activity to the presence of these chlorinated chlorosulfolipids in the O. danica extracts (10-13). Fluoroacetic acid is another halogenated natural product with which I am sure you are familiar (Fig. 5). Marais discovered fluoroacetate as the toxic principle in the leaves of an African plant (14). Peters subsequently showed that fluorinated longer chain fatty acids could also be found in nature (15-17). Finally, from the marine field, I have selected one compound to call to your attention. Laurinterol (Fig. 6) is a brominated terpene produced by red algae, especially by a number of Laurencia species (18). Laurinterol has very potent antibiotic activity. It is comparable to penicillin in terms of its range and its antimicrobial activity.

About seven years ago, in collaboration with Ken Rinehart and Paul Shaw, my laboratory undertook a survey of halometabolites in marine organisms. We randomly collected about 1200 different species

$F-CH_2-COO^-$

Fluoroacetate (first isolated from leaves of Dichapetalum cymosum)

Fluorooleate (isolated from seeds of Dichapetalum toxicarium)

Figure 5. Fluorinated natural products.

Figure 6. Laurinterol.

of marine organisms from the coastal waters of Baja, California and analyzed them for their organic halogen content. We also analyzed the extracts we obtained from these organisms to see whether there was any correlation between antimicrobial activity and their halogen content. To make a long story short, there was a strong correlation between the presence of organic halogens or halometabolites in the organisms and their ability to inhibit the growth of other organisms as shown in Figure 7. This survey was carried out using the research vessel, the Alpha Helix, a National Science Foundation Facility which, unfortunately, is no longer available for biological research. The results of our halometabolite survey is briefly summarized in Table I. There were some 656 different animal species and about 186 plant species examined in detail during that investigation. The average concentration of halometabolites in these organisms expressed in terms of brominated and chlorinated molecules is shown in Table I, where halometabolite levels are expressed in terms of micrograms of halometabolite per gram wet weight of tissue. In order to give you some idea what the numbers in Table I mean, an average value of 10 micrograms of organic halogen per gram wet weight of tissue indicates that about 1 out of every 100 lipid molecules extracted from the tissue of the organism contained either a chlorine or bromine atom. When that number rises to 100 micrograms of halogen per gram of wet weight, there are about 10 molecules of halogen per 100 lipid molecules or, in other words, 10% of the lipid

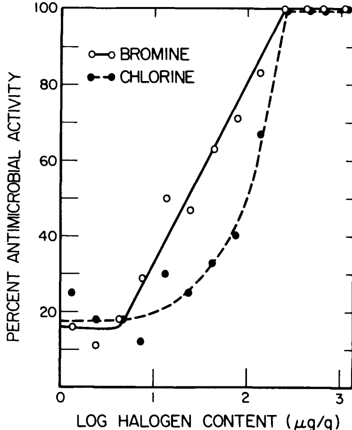

Figure 7. Correlation of halometabolite levels with antimicrobial activity.

TABLE I
Halometabolite Levels in Marine Organisms

Kingdom	Number of Species Examined	Average Halometabolite Concentrations: Chlorometabolites µg/g wet weight	Bromometabolites µg/g wet weight
Animal	656	9.6	5.1
Plant	186	19.8	19.3

Marine Species Containing High Levels of Halometabolites

Scientific Name	Type	Lipid Halometabolite Concentration µg/g wet weight
Verongia aurea	Sponge	700
Muricantha princeps	Rock Shell	50
Tylodina fungia	Nudibranch	600
Ophioderma variegatum	Brittle Star	68
Asparagopsis taxiformis	Red Algae	2400
Plocamium pacificum	Red Algae	1140
Laurencia masonii	Red Algae	530

molecules are halogenated. When the halogen concentration goes as high as 1,000 µg per gram of tissue, on the average every lipid molecule contains a halogen atom. You will note that the average value for the plants is fairly high in Table I ; twenty µg of chlorine and 20 µg of bromine per gram of algal tissue. Thus, on the average, about 20% of the lipid molecules in marine plants contained halogen atoms. In some cases, the halometabolite levels rise to really astronomic levels. For example, in some selected red algae species such as Asparagopsis, we find bromine levels of 2,400 µg per gram of tissue. That calculates out on the average as 2.4 atoms of bromine for every lipid molecule present in the organism. Translated into halometabolite concentrations on a dry weight basis, about 5% of the dry weight of the organism consists of organic compounds. When we started our survey of marine halometabolites, there were very few halometabolites of marine origin known, perhaps a dozen to 20 examples. However, there has been considerable activity in the marine halometabolite field during the past 7 years. There are now over 700 marine halometabolites which have been isolated and characterized. The marine halometabolites fall roughly into 5 major classes. These classes consist of the halogenated hydrocarbons, the halogenated ketones, halogenated phenolic and terpenes. Finally, there is a separate family of ^{15}C non-terpenoid compounds.

One of the compounds from the halogenated hydrocarbon family is

a very unusual product. It was originally discovered as a consequence of the concern over the presence of halohydrocarbons in the upper atmosphere, especially concern about fluorinated halohydrocarbons, their reaction with ozone, and possible destruction of the ozone layer. This concern triggered considerable research on the concentration and composition of halohydrocarbons in the upper atmosphere. Monochloromethane (methyl chloride) turns out to be the predominant species of halohydrocarbon in the upper atmosphere and it does not originate from industrial pollution (19). Monochloromethane arises from biological activity in sea water. The concentration of monochloromethane is highest in and around tropical ocean waters and is especially abundant over large algae beds. Since the steady state level of monochloromethane in the upper atmosphere has been determined and since one can calculate a rate for the destruction of monochloromethane by its reaction with ozone and other relative species in the upper atmosphere, it is relatively simple to calculate an annual global emission rate required to maintain the steady state level. The annual global emmission rate is conservatively estimated to be 2×10^7 tons per year. That is a lot of gas. In contrast to thyroxine which is maintained at steady state levels of 1×10^{-9} molar, we now have 2 trillion tons of a halohydrocarbon being synthesized by marine algae, excreted into ocean waters and eventually diffusing into the upper atmosphere. Unfortunately, we do not have any idea at the present time why marine algae like to make volatile halohydrocarbons.

I would now like to turn to a brief discussion of the enzymes which are responsible for the insertion of chlorine or bromine atoms into organic molecules. The organism we chose for our initial study of enzymatic halogenation was the fungus, *Caldariomyces fumago*. This organism was one of the five organisms that Raistrick had found to be a high incorporator chloride ion (5). When you put a gram of chloride ion into one liter of growth medium and innoculate it with *C. fumago*, you obtain about 0.2 gram of chlorine incorporated into a single product, 2,2-dichloro-1,3-cyclopentanediol. The trivial name of caldariomycin has been assigned to this structure. We chose this organism for our initial study because this natural product has a simplicity of structure that is not found in most other halogenated compounds of natural origin. We thought this structural simplicity might simplify the task of finding the enzyme that would catalyze the insertion of chlorine atoms into this structure. Paul Shaw in my laboratory was the first to detect a chlorinating enzyme activity in *C. fumago* (20). The chlorinating enzyme has subsequently been purified, characterized, and crystallized (21). The properties of chloroperoxidase are briefly summarized in Table II. It will be noted that chloride, bromide, and iodide very efficiently oxidized in the presence of chloroperoxidase. The turnover number indicates that 1 molecule of enzyme will oxidize 66,000 molecules of chloride or 250,000 molecules of iodide per minute.

TABLE II
Properties of Chloroperoxidase

1.	Prosthetic Group: ferricrotoporphyrin IX
2.	Molecular Weight: 40,000 - 42,000
3.	Carbohydrate Content: ~ 25%
4.	Principal Sugar Residues: glucose, galactose and mannose
5.	Turnover Number: 66,000 Cl^-; 120,000 Br^-; 250,000 I^-

The overall halogenation reaction has as an intermediate step - the oxidation of a halide anion by hydrogen peroxide. This oxidation generates an enzyme-bound electrophilic halogenating species which will react with a variety of acceptor molecules to form halogenated products. A large variety of acceptors will function in this reaction. Cyclic β-diketones, β-ketoacids, anisole, phenol, and tyrosine will all function as halogen acceptors. Thus, the enzyme is relatively non-specific with respect to the halogen acceptor. A rather unique use for this enzyme is being developed by Cetus Corporation in cooperation with Cheveron Oil Company. Cetus is developing an enzymatic route for the synthesis of epoxides as outlined in Figure 8. This process uses an olefin as starting material and generates the corresponding epoxide as a product. The process also produces a co-product since hydrogen peroxide must be provided in order to carry out the intermediate formation of the chlorohydrin. The Cetus plan generates fructose as a second product which arises from the oxidation of glucose to glucosone with the subsequent non-enzymatic reduction of glucosone to fructose. Relatively large amounts of a halogenating enzyme, either chloroperoxidase or a similar haloperoxidase will be required in order to make this an efficient process. Figure 9 describes our fledgling start on the molecular biology of large scale chloroperoxidase production. Chloroperoxidase itself is a typical secondary mold metabolite. That is,

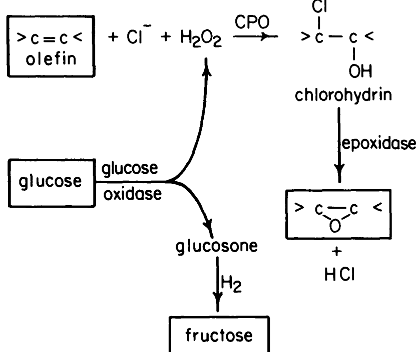

Figure 8. Cetus process for enzymatic synthesis of epoxides.

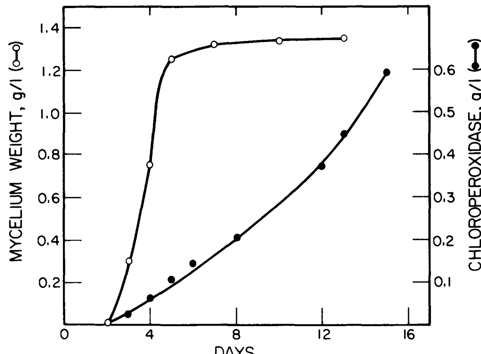

Figure 9. Growth of Caldariomyces fumago and chloroperoxidase excretion.

the organism, C. fumago, grows to essentially stationary phase without producing chloroperoxidase. However, after stationary phase growth is achieved, C. fumago starts manufacturing odd compounds like caldariomycin. Not unexpectedly, chloroperoxidase synthesis and excretion coincides with the formation of those secondary products. The amazing thing about the synthesis and excretion of chloroperoxidase is the large amount of enzyme which is produced. Conditions can be arranged so that one-third of the total protein in the culture can be accounted for in terms of the halogenating enzyme, chloroperoxidase. Figure 9 shows that after about 5 or 6 days of chloroperoxidase excretion, there is approximately 650 mg per liter of protein in the mycelium of the organism and about 300 milligrams per liter of chloroperoxidase excreted into the culture medium, essentially 100% pure. Thus, C. fumago is a magnificent machine for producing a single protein and excreting it into the culture medium, essentially as a pure protein. We would like to take advantage of this unique production medium and use it to produce proteins other than chloroperoxidase. It should be possible to take advantage of C. fumago and its regulatory genes in the following way. Presumably there is a promoter sequence in C. fumago DNA which turns on mRNA synthesis from the chloroperoxidase structural gene in stationary phase cells. There would also have to be some kind of DNA signal sequence dictating that this enzyme is to be excreted into the culture medium. We should be able to manipulate these two DNA sequences so that other structural genes could be tied to these signal and promoter DNA sequences. If successful, C. fumago could be used to synthesize and excrete a vareity of pure enzymes and proteins. Unfortunately, the genetics of Caldariomyces fumago remain largely unknown. So considerable preliminary work will have to be done before recombinant DNA techniques can be applied to this organism.

R1	R2	R3	R4	No.
Br	H	Br	H	72
Br	Br	Br	H	73
Br	Br	Br	Br	74
Br	H	Cl	H	75
Br	Br	Cl	H	76
Br	Cl	Br	H	77
Br	Br	Br	Cl	78

R1	R2	No.
Br	H	79
Cl	H	80
Br	Cl	81
Br	Br	82

Figure 10. Scheme for bromoform, dibromomethane and pentyl bromide synthesis.

I would like to close by discussing very briefly the biosynthesis of one of the volatile halohydrocarbons found in the upper atmosphere. Bromoform is one of the principle brominated compounds found in those red algae which accumulate large amounds of brominated compounds (22). Along with bromoform, these algae also accumulate halogenated ketones of the type illustrated in Figure 10. When extracts from these organisms, for example _Bonnemaisonia hamifera_ extracts, are incubated with suitable precursors, brominated ketones and bromoform are synthesized. 3-Ketooctanoic acid was considered as an obvious precursor because the insertion of a bromine atom followed by decarboxylation would lead to the whole family of brominated ketones shown in Figure 10. Table III shows that extracts of _B. hamifera_ do carry out the biosynthesis of these brominated ketones from 3-ketooctonoic acid. These extracts will also catalyze the formation of bromoform from 3-ketooctanoic acid and bromide ion (24). Bromopentane and dibromomethane have also been detected as products of the enzyme reaction (24). We visualize these products to be formed from the corresponding brominated ketone through a series of enzymatic hydrolysis steps as outlined in Figure 11. My laboratory is now heavily engaged in the isolation and characterization of bromoperoxidases from marine algae. We hope that our studies will eventually shed light on both the reasons of why and how these halogenated compounds are synthesized by marine organisms. Right now, it seems that we know that Mother Nature chooses to make a large variety of halogenated compounds especially in marine organisms where there is obviously an ecological niche for halogenating

TABLE III
Bromoform and Bromoheptanone Synthesis from Various Substrates

substrate added	condition[a]	products formed or substrate recovered (nmol)				
		monobromoheptanone	dibromoheptanone	tribromoheptanone	$CHBr_3$	total
monobromoheptanone	a	1960	168	112	479	2719
	b	8456	0	0	0	8456
	c	3108	0	0	0	3108
	d	7658	0	0	0	7658
dibromoheptanone	a	0	406	63	1113	1582
	b	0	1400	0	0	1400
	c	0	1666	0	0	1666
	d	0	2310	0	0	2310
tribromoheptanone	a	0	266	1673	910	2549
	b	0	126	420	882	1428
	c	0	189	861	1274	2324
	d	0	70	70	623	763
β-oxooctanoic acid	a	357	133	77	1260	1827
	b	0	0	0	0	0
	c	0	0	0	0	0
		0	0	0	0	0

[a]The experimental conditions were as follows: (a) the complete system which contained 50 mM potassium bromide, 100 mM potassium phosphate-citrate buffer, 1 mM halogen acceptor substrate, 0.7 units/ml of bromoperoxidase and a steady state level of approximately 1 mM H_2O_2, (b) complete system minus the enzyme, (c) complete system minus H_2O_2, (d) complete system minus enzyme and H_2O_2.

$CH_3-(CH_2)_3-CH_2-C(=O)-CH_2-C(=O)-OH$ (R = $CH_3-(CH_2)_3-CH_2$)

3-ketooctanoic acid

Enz-Br$^+$ → $CH_3-C(=O)-OH$ + $CH_3-(CH_2)_3-CH_2Br$ (1-bromopentane)

Enz-Br$^+$ → $R-C(=O)-CH_2Br + CO_2H$

Enz-Br$^+$ → $R-C(=O)-CHBr_2$ → $R-C(=O)-OH + CHBr_2$ (dibromomethane)

Enz-Br$^+$ → $R-C(=O)-CBr_3$ → $R-C(=O)-OH + CHBr_3$ (bromoform)

Figure 11. Formation of bromopentane and dibromomethane from 3-ketooctanoic acid enzyme reaction.

enzymes. Since ocean waters are 0.5 M in chloride ion and 1 x 10^{-5} M in bromide ion, there are plenty of halogen anions to insert into organic molecules. I think that many new and interesting compounds will show up as our work progresses in that field.

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DISSCUSSION

Q. I think these two papers really represent a study in contrast; on the one hand, Mother Nature has invented a single system for making methane and packaged it in a small group of microbes called methanogens. This system, apparently, is not in equilibrium with any of the rest of biology. On the other hand, the chlorinated and brominated compounds seem to be everywhere - from man to plants to microbes. So what do they really do for people?

A. HAGER: Well, I think that most of the halogenated compounds that we have studied serve as protective devices for the organism. For example, the marine algae grow under conditions where they are subjected to mechanical damage due to wave action. It would be advantageous for them to keep around some good antibiotics to protect themselves from attack by marine bacteria. Why marine algae would choose to synthesize trillions of tons of monochloromethane is beyond me at this time. I do not have any ideas.

Q. Does light play any role in the release of these halogenated compounds? Is more released in the daytime than at night?

A. HAGER: I don't know and I don't think that has been studied. The studies on the halohydrocarbons in the upper atmosphere are done by collecting the gases after they have diffused into the upper atmosphere. There would not be any way to titrate that data back to some kind of cyclic day-night production.

Q. JUNI: Can chloromethane be used as a fuel?

A. HAGER: Well, it would not be a very good fuel. As you replace hydrogen atoms with halogens you lose combustability. As you know, carbon tetrachloride is used to put out fires.

Q. BERGMAN: Is it known how fluorine is introduced into natural products?

A. HAGER: It cannot be introduced by the same mechanisms I have described for chlorination, bromination, or iodination. It is, of course, impossible to oxidize fluoride ion with hydrogen

peroxide. Thus, fluoride must probably be introduced into natural products as fluoride ion. We have made some feeble attempts to study enzymatic fluorination but we have not made much progress.

Q. If I understand correctly, you suggest that chloroperoxidase is synthesized in the stationary phase. Did you check for the presence of the apoprotein in the cell during early growth?

A. HAGER: We cannot detect enzyme activity by breaking open the cells and preparing cell extracts from cells in the logarighmic growth phase. In addition, we cannot detect homo- or apo- enzyme from stationary phase cell free extracts. If apoenzyme is formed and stored inside the cells, we are unable to reconstitute it. We have added heme that would allow conversion to holoenzyme, but we do not detect activity. The only place we have seen activity is in the culture medium, never inside the cell.

GENETICS AND BIOCHEMISTRY OF TYLOSIN PRODUCTION: A MODEL FOR GENETIC ENGINEERING IN ANTIBIOTIC-PRODUCING *STREPTOMYCES*

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INTRODUCTION

Antibiotics are economically-important chemicals produced by a variety of species of prokaryotic and eukaryotic microorganisms. Species of the prokaryotic genus, *Streptomyces*, produce about 70 percent of all known antibiotics (19). Therefore, the *Streptomyces* present a practical model for genetic manipulation. *Streptomyces* are also fundamentally interesting for at least two reasons. First, they undergo a differentiation cycle of spore germination, mycelial growth, aerial mycelium formation and sporulation (17). In general, the relationships between the differentiation cycles and antibiotic production are only poorly understood. Second, the *Streptomyces* contain circular genomes (17,19) with guanine plus cytosin (G+C) contents of 69 to 73 percent (49). This G+C content approaches the genetic code upper limit (50), beyond which the amino acid content of proteins would have to deviate substantially from the bacterial norm. Since the latter is highly unlikely (47), the *Streptomyces* must have severely constrained codon usage patterns which should maximize the G+C content in the third position of codons. It is not known if there are additional constraints in noncoding sequences which, for instance, might affect promoter sequences.

Also, the evolutionary significance of very high G+C content and its consequences with regard to spontaneous and induced mutagenesis, and DNA replication and repair mechanisms remain obscure. Thus, the apparent genetic code limitations, differentiation and antibiotic production make the *Streptomyces* particularly attractive microorganisms for future molecular biological research and genetic manipulation.

Prior to 1977, the only methods available for genetic manipulation of *Streptomyces* were mutation and conjugation (12,17,19,37). Unfortunately, many species of commercial interest were either only marginally fertile or non-fertile (2,19,37). However, in the past four years two very important techniques for genetic manipulation have been developed and refined. The first is protoplast fusion (1-4,15,21-23,28) to achieve remarkably high frequencies of genetic recombinants. The second is protoplast transformation (9) and transfection (45) which have enabled rapid development of gene cloning in *Streptomyces* (8,10,20,40,46,48). These techniques have provided the means for facile genetic analysis and manipulation, and should aid in both fundamental and applied studies with a wide variety of *Streptomyces* species.

In this report, I will discuss two general applications of genetic engineering in *Streptomyces*. These are genetic manipulation of antibiotic yield, and formation of heterospecific recombinant strains to produce novel antibiotics. Rather than survey a wide variety of *Streptomyces* species and antibiotic structures, I have chosen to focus very narrowly on one species, *Streptomyces fradiae* and one specific antibiotic class, macrolides, since general applications can be extrapolated from these.

GENETICS AND BIOCHEMISTRY OF TYLOSIN PRODUCTION

Tylosin is a 16-membered macrolide antibiotic produced commercially by a strain a *S. fradiae* (43). It is also produced by *Streptomyces rimosus* (36) and *Streptomyces hygroscopicus* (24). Tylosin is composed of a branched lactone (tylonolide) and three sugars, mycarose, mycaminose and mycinose (Fig. 1). We have been interested in understanding the genetics and biochemistry of tylosin production, and in applying a variety of genetic techniques to manipulate tylosin yield. In addition, we have been interested in identifying tylosin intermediates or shunt metabolities which might be biologically or chemically converted to additional novel antibiotic structures. To aid these studies, we have isolated a series of mutants blocked in specific tylosin biosynthetic steps (5).

Table I summarizes structures of various tylosin intermediates and shunt metabolities produced by certain *tyl* mutants (5). The *tyl* mutants have also been analyzed in cofermentation, *in vivo* bioconversion and *in vitro* bioconversion experiments (5,6,7,41,42) to determine the sequence of biosynthetic steps. Fig. 2 summarizes the most likely biosynthetic pathway to tylosin and indicates the positions of genetic blocks (i.e., A, B, C, etc.). It also shows the biosynthetic relationships between the shunt metabolities produced by various blocked mutants. The first intermediate excreted is tylactone (Fig. 1 and 2; Table I) which has methyl

TYLACTONE

TYLOSIN

MYCINOSE

MYCAMINOSE

MYCAROSE

Figure 1. Structures of tylactone and tylosin.

groups at the C-20 and C-23 positions. It was shown previously that the lactone portion of tylosin is derived from two acetate units, five propionate units and one butyrate unit (34,35). Since condensation of the lactone appears to occur by a mechanism analogous to that of long chain fatty acid biosynthesis (31), tylactone is probably formed from one propionyl CoA, four methyl-malonyl CoA's, two malonyl CoA's and one ethyl-malonyl CoA (5). Thus, the C-19, C-20 and C-23 positions are probably derived from the ethyl- and methyl- groups of ethyl-malonyl CoA and methyl-malonyl CoA, respectively. Tylactone appears to be converted to tylosin by a preferred sequence of reactions (5,6,7,40,41,42) which include: 1) addition of mycaminose to the 5-OH position; 2) oxidation of C-20 methyl to hydroxymethyl; 3) oxidation of C-20 hydroxymethyl to formyl; 4) oxidation of C-23 methyl to hydroxy-methyl; 5) addition of 6-deoxy-D-allose to C-23 hydroxyl; 6) addition of mycarose to 4'-OH of mycaminose; 7) O-methylation of the 2'''-OH of the 6-deoxy-D-allose moity of demethylmacrocin; and 8) O-methylation of the 3'''-OH of the 3-demethylmycinosyl moiety of macrocin to produce tylosin. Note that only two of the steps beyond tylactone formation cannot be bypassed in blocked mutants (Figure 2 and Table I). These are addition of mycaminose to tylactone (blocked in *tylA* and *tylB* mutants) and O-methylation of the 2'''-OH position (blocked in the *tylE* mutant), Since the oxidation reactions and neutral sugar additions can be bypassed,

TABLE I

Structures of Tylosin Precursors and Shunt Metabolites

	Compound	Oxidation Level		Sugars Present			O-methyl Groups Present		Precursor (P) or Shunt Metabolite(S)	Producing Strains[b]
		C20	C23[a]	Mycaminose	Mycarose	6-deoxy-D-allose	2'''	3'''		
1	Tylactone	CH_3	CH_3	-	-	-	-	-	P	TylA or TylB
2	O-mycaminosyl tylactone	CH_3	CH_3	+	-	-	-	-	P	-
3	20-deoxy-20-dihydro-O-mycaminosyl tylonolide	CH_3	CH_2OH	+	-	-	-	-	S	TylI, TylD[c]
4	20-dihydro-23-deoxy-O-mycaminosyl tylonolide	CH_2OH	CH_3	+	-	-	-	-	P	-
5	23-deoxy-O-mycaminosyl tylonolide	CHO	CH_3	+	-	-	-	-	P	-
6	O-mycaminosyl tylonolide	CHO	CH_2OH	+	-	-	-	-	P	-
7	20-deoxy-20-dihydro-demycinosyl tylosin	CH_3	CH_2OH	+	+	-	-	-	S	TylI, TylD[c]
8	23-deoxy-demycinosyl tylosin	CHO	CH_3	+	+	-	-	-	S	TylH
9	Demycinosyl tylosin	CHO	CH_2OH	+	+	-	-	-	S	TylD
10	Demethyllactenocin	CHO	CH_2OR	+	-	+	-	-	P	-
11	Demethylmacrocin	CHO	CH_2OR	+	+	+	-	-	P	TylE
12	Lactenocin	CHO	CH_2OR	+	-	+	+	-	S	-
13	Macrocin	CHO	CH_2OR	+	+	+	+	-	P	TylF
14	Desmycosin	CHO	CH_2OR	+	-	+	+	+	S	TylC
15	Tylosin	CHO	CH_2OR	+	+	+	+	+	-	Wild Type

[a] R refers to 6-deoxy-D-allose or methylated derivatives.

[b] These are mutant strains containing specific genetic blocks in tylosin biosynthesis.

[c] This is a double mutant.

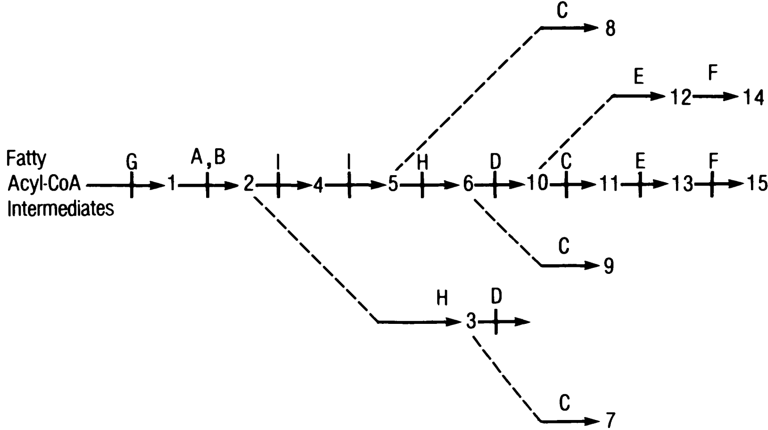

Figure 2. Biosynthetic relationships between tylosin intermediates and shunt metabolites produced by various blocked mutants. The numbers refer to the macrolide compounds described in Table I. The letters refer to specific genetic blocks (i.e., tylA, tylB, etc.) and dashed lines indicate the specific steps bypassed in certain blocked mutants.

a variety of shunt metabolites have been readily identified (5). Also, additional biologically-active macrolide compounds are readily produced by chemical reduction of the C-20 formyl group to hydrxy-methyl, and by mild acid cleavage of mycarose (6,7).

We have begun to analyze the genetic map locations of the tylosin genes by protoplast fusion (1,2,3,4) and conjugation. Preliminary experiments suggest that many of the tylosin genes may be located on a self-transmissible genetic element (6).

GENETIC ENGINEERING FOR YIELD IMPROVEMENT

The two general applications for genetic engineering in antibiotic-producing Streptomyces are for yield improvement and for production of novel antibiotics. Before one can rationally exploit genetic engineering for yield improvement, one first needs to define the crucial factors controlling antibiotic productivity. A key question, then, is whether the intermediates from primary metabolism, cofactors, etc., or antibiotic-specific enzyme levels control the rates of antibiotic biosynthesis. We have attempted to answer this question with regard to tylosin production in S. fradiae by measuring the levels of a tylosin-specific enzyme, S-adenosyl-L-methionine: macrocin O-methyltransferase, in a series of mutants selected for

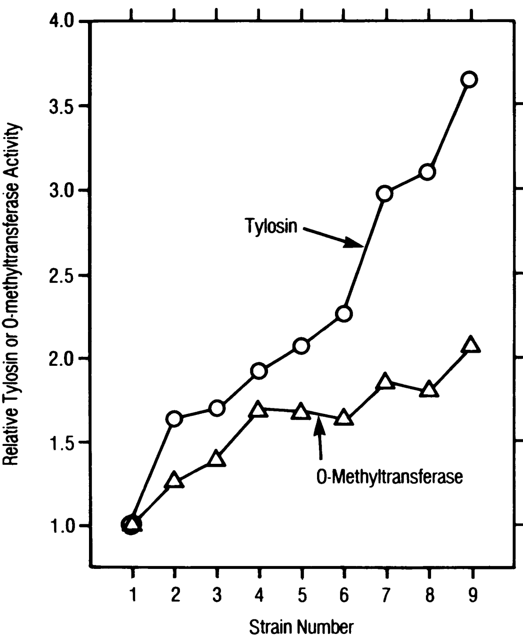

Figure 3. Relative tylosin production and macrocin O-methyltransferase activities in a series of S. fradiae mutants selected for increased tylosin production. Tylosin concentrations were determined at the end of a normal fermentation cycle. Macrocin O-methyltransferase specific activities were determined at five points in the fermentation and averaged.

progressively increased tylosin production (41,42). For this analysis, we have assumed that if the tylosin biosynthetic enzymes are regulated in a concerted manner, the level of macrocin O-methyltransferase in particular strains may be indicative of the general levels of expression of other tylosin biosynthetic enzymes. (This assumption has not yet been confirmed by independent assay of other tylosin enzymes.) The rationale of the analysis, then, is that if precursor levels, cofactors, etc., are the limiting factors for tylosin biosynthesis rather than antibiotic-specific enzymes, then the specific activity of macrocin O-methyltransferase should remain relatively constant throughout the series of strains. If, on the other hand, tylosin-specific enzyme levels control the rate of tylosin biosynthesis, then one should observe progressively higher levels of macrocin O-methyltransferase associated with higher tylosin production in mutant strains of S. fradiae. We have found (41,42) in several cases that increased tylosin production is accompanied by increased specific activity of macrocin O-methyltransferase (Figure 3). However, in other cases, enhanced tylosin

production was apparently not associated with increased macrocin O-methyltransferase. Therefore, the data suggest that both tylosin-specific enzyme levels and other factors such as precursor levels can be limiting, depending on the strain in question, and that highly developed strains may require dual genetic alterations to enhance the expression of antibiotic-specific enzymes and the levels of precursors, cofactors, etc. to ensure additional large increases in antibiotic productivity. Cloning can be directed toward enhanced expression of tylosin-specific genes, while more classical genetic techniques (12,37) can be used to enhance the levels of tylosin-specific precursors.

A relatively straight-forward application of gene cloning is to amplify a gene which codes for a rate-limiting enzyme. An example in tylosin biosynthesis is the macrocin O-methyltransferase gene. Even though highly productive strains of *S. fradiae* produce elevated levels of macrocin O-methyltransferase (Figure 3), it is somewhat rate-limiting in these strains (42). We have shown that the enzyme is inhibited by its substrate, macrocin, and its product, tylosin, and by other tylosin intermediates and shunt products which contain mycinose, demethylmycinose or 6-deoxy-D-allose attached at the C-23 position (41 and Table I). The apparent K_I for all inhibiting macrolides was about 60 μM, which is well below the concentrations of tylosin excreted during normal fermentations. Therefore, we suspect that during the late stages of tylosin fermentation in highly productive strains of *S. fradiae*, the intracellular levels of certain macrolides reach inhibiting concentrations. This apparent rate limitation might be relieved by increasing enzyme concentrations by cloning and amplifying the gene copy number. In addition, the cloned O-methyltransferase (*tylF*) gene might be genetically altered *in vitro* by site-directed mutagenesis to increase the enzyme K_I (3).

GENETIC ENGINEERING FOR NOVEL ANTIBIOTICS

Many different macrolide antibiotics have been identified (33), and some of the biosynthetic steps show less than absolute substrate specificities. Therefore, certain macrolide-producing microorganisms can carry out biological conversions of other macrolide antibiotics with structures similar to those normally produced (14,27,29,30,32). This two-stage fermentation-bioconversion approach is limited to compounds which are excreted, relatively stable, and readily taken up by bioconverting microorganisms. One could improve on this approach by isolating mutants which produce a variety of different intermediates and shunt metabolities (such as with the tylosin pathway) for bioconversion, and then introducing genes coding for specific bioconverting enzymes into the mutant strains. With this approach, one can envision the possibility of producing large

Figure 4. Potential biological modifications of tylosin. The sugar stereochemistry has been distorted somewhat to facilitate certain projections (see Figure 1).

numbers of novel macrolide antibiotic structures which might be further modified chemically. Consider the tylosin system as an example. Figure 4 shows a number of modifications of the tylosin structure potentially attainable by mutational blocks and bioconversions. For convenience, O-mycaminosyl tylactone is shown as the least complex structure.

The C-23 position of O-mycaminosyl tylactone is a methyl group which is normally oxidized to hydroxymethyl before addition of 6-deoxy-D-allose (see Figures 1 and 4). After attachment, this sugar is converted to mycinose by two specific methylations. Thus, mutations blocking these four biosynthetic steps (i.e., _tylH_, _tylD_, _tylE_ and _tylF_) define five potential chemical structures at C-23.

Certain Streptomyces species can convert the 12, 13-double bond to an epoxide (33). Therefore, two possible configurations exist at this position. Also, certain Streptomyces reduce the C-9 ketone function to hydroxyl (33), and S. ambofaciens can carry out this reaction with tylosin (32). S. ambofaciens further modifies this position by addition of forosamine during normal biosynthesis of spiramycin (33). Thus, three potential configurations exist at C-9.

The C-20 position of O-mycaminosyl tylactone is a methyl group which is converted to a formyl group in two steps (5,6,7). The formyl group is readily converted to a hydroxymethyl group by a number of actinomycetes (5,14) or by chemical reduction. Thus, the C-20 position can exist in three different configurations.

Mycaminose is added to the C-5 position of tylactone during tylosin biosynthesis. However, certain other Streptomyces or Micromonospora species can attach other amino sugars to similar macrolide structures (33). These sugars, angolosamine and desosamine, are 2-deoxy or 4-deoxy analogues of mycaminose, respectively. The amino sugars containing 4'-hydroxyl (i.e., mycaminose or angolosamine) can be modified by addition of mycarose (33). This reaction is blocked in S. fradiae tylC mutants. Mycarose can be further modified by certain macrolide-producing Streptomyces by four different acylations at the 4"-position (33), and several of these bioconversions have been demonstrated with tylosin (29,30).

The C-3 position of the lactone can undergo two different acylations (33) and both bioconversions have been demonstrated with tylosin (29,30). Also, certain streptomycetes dehydrate their macrolide products at the C-2, C-3 position (33,39).

From the foregoing example, it is clear that a wide variety of macrolide antibiotic structures might be produced by cloning specific genes into specific blocked mutants. In the case of tylosin, well over 1,000 novel structures can be envisioned. Coupling this approach with chemical modifications in vitro could potentially lead to very large numbers of novel antibiotic structures. This poses an interesting question as to which compounds might be most useful, since one cannot practically produce all possible combinations. Thus, application of classical and recombinant DNA genetics to novel antibiotic production will inevitably require close scrutiny of structure-function relationships.

The foregoing discussion has been presented only as an example of the possibilities of genetic engineering. This approach should be applicable to other antibiotic structures, but it presumes a certain degree of knowledge of the genetics and biochemistry for

the particular antibiotic structures of interest and presumes that gene cloning will become available in a variety of Streptomyces species.

FUTURE PROSPECTS

The highly efficient protoplast techniques for genetic recombination (1-4,15,21-23,28) and gene cloning (8,9,10,20,40,46,48) have dramatically increased the possibilities for genetic manipulation of the antibiotic-producing Streptomyces. These techniques should facilitate the probing of fundamental questions on the structure and regulation of antibiotic genes. For instance, with few noted exceptions (18,19), little is known about the genetic locations (plasmid or chromosomal) and organization of antibiotic genes, or how antibiotic genes are regulated at the levels of transcription and translation. Also, from a technical point of view, many plasmids have been identified in Streptomyces (16,18), but only two have been developed for gene cloning (8,10,20,40,48). One vector appears to have fairly wide host specificity, however (20). Only one actinophage cloning vector is available (46), but it may have limited host range (11). Also, many Streptomyces produce restriction endonucleases (11,13,26,38,44), and many of these enzymes function to restrict actinophage DNA *in vivo* (11,26). Thus, additional fundamental work on plasmids, actinophages, and restriction/modification systems in Streptomyces will probably accelerate practical applications of gene cloning in this economically important genus. In addition, it is likely that most, if not all, antibiotic resistance mechanisms discovered in other genera will have counterparts within particular Streptomyces. In spite of this, no endogenous translocatable drug resistance element has yet been harnessed for genetic engineering in Streptomyces. Thus, fundamental studies on transposons in Streptomyces would also be useful to establish this powerful methodology for genetic engineering in this economically important genus (25,37).

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DISCUSSION

Q. GARWIN: Do you think that it might be possible to use confermentation of different actinomycetes to obtain novel antibiotics? For instance, to obtain modifications of tylosin-like molecules.

A. BALTZ: That might be possible, particularly with strains which produce similar types of antibiotics. However, *Streptomyces* and other actinomycetes often inhibit each other in liquid growth medium. You might get around this by giving one strain an advantage, perhaps by allowing it to grow for some time before adding the second strain.

ROUNDTABLE DISCUSSION OF RESEARCH PRIORITIES

The purpose of this panel is to discuss some future research needs and priorities in the area of genetic engineering of microorganisms.

Participants

Raymond C. Valentine (Chairman)
University of California, Davis

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The Upjohn Company
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The authors and editors have expanded or condensed their remarks with questions and answers for presentation in this publication.

POTENTIAL FOR GENETIC ENGINEERING OF HYDROGEN BACTERIA *

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Hydrogen bacteria constitute a diverse group of microorganisms able to grow autotrophically in simple mineral media utilizing the gaseous substrates H_2, O_2, and CO_2. Hydrogen gas is a major, cheap feedstock in the chemical industry (e.g., for ammonia synthesis). It is mostly produced from fossil fuel, but can also be produced from renewable sources such as biomass. Hydrogen may therefore represent an alternative to carbohydrate as an energy source for new fermentation processes to make products such as single cell protein, amino acids and other fine chemicals, enzymes, etc. Use of H_2 as a feedstock would have certain advantages over carbohydrate, currently the major substrate for industrial fermentations. The gaseous substrates can be delivered with high purity, are easy to sterilize and leave no residues. Hydrogen bacteria normally do not make any metabolic waste products other than water. This would provide a very clean fermentation system, which would simplify product isolation and purification.

Effective harnessing of H_2 bacteria for production of useful products depends on the development of both *in vivo* and *in vitro* systems for genetic manipulations of these organisms, in addition to a thorough understanding of their physiology and biochemistry. The discussion below will show that significant progress is now being made in these areas. During the past two decades, H.G. Schlegel and coworkers have pioneered comprehensive studies of the general microbiology, physiology, and biochemistry of hydrogen bacteria, especially *Alcaligenes eutrophus* (1). Almost all H_2 bacteria are facultative autotrophs capable of growth on many organic substrates. They are strictly aerobic, some can also respire with NO_3^-. Ammonia or urea may serve as nitrogen source. Several new N_2-fixing strains have also been isolated (20). Most

strains grow best at 30-40°C, however, thermophilic strains have recently been isolated in Japan (3).

A. eutrophus is simple to cultivate and is among the fastest growing H_2 bacteria with generation time 2-3 h under autotrophic conditions, and it can be grown to extremely high cell densities (exponential growth to 19 g dry weight cells per 1 (4)). This organism therefore has potential for high rates of product formation in small fermenters. Its energy efficiency of growth is also high, reaching 45% under O_2-limitation (5). NASA has sponsored studies of the cultivation of H_2 bacteria using electricity as energy source, work based on the potential of these organisms as a foodstuff for travelers in deep space (6). *A. eutrophus* makes single cell protein of high nutritional quality (7), and this could make a useful byproduct of fermentation processes involving this organism. Many H_2 bacteria, including *A. eutrophus*, accumulates large quantities of poly-β-hydroxybutyric acid (PHB) when starved for nitrogen. PHB is a biodegradable polyester with many properties similar to polypropylene, and it is being explored as a possible plastic substitute (8).

A. eutrophus fixes CO_2 through the Calvin cycle and was reported to possess a plant-type ribulose bisphosphate carboxylase (RuBPCase) (9,10). This prompted our isolation of a comprehensive set of CO_2 fixation mutants including RuBPCase structural gene mutants as a model system for this critical enzyme in green plants (11). A close correlation between the carboxylase and oxygenase function of RuBPCase was found in these studies involving mutant forms of RuBPCase. To date, mutants with more efficient carboxylase relative to oxygenase activity have not been found. A study of the efficiency of CO_2 fixation *in vivo* was carried out using mutants blocked in the oxidation of glycollate (12). As much as one-half of the fixed carbon was found to be excreted as glycollate when the CO_2/O_2 ratio was least favorable. Such studies provide the basis for maximizing the CO_2 fixation system of H_2 bacteria for production of useful chemicals. The induction of very high levels of RuBPCase as well as hydrogenase has recently been achieved, using suitable alterations of the growth conditions (11, unpub.).

Hydrogen metabolism by *A. eutrophus* has been extensively studied by Schlegel and coworkers. This organism has two pathways for H_2 utilization (13). Both the soluble and the membrane-bound hydrogenase have been purified and characterized (14,15), and mutants blocked in H_2 utilization have been studied (16). We have recently identified a plasmid in *A. eutrophus* which governs the utilization of H_2 as a sole source of energy and reductant (17). Cured strains regain the ability to use H_2 following conjugal transfer of this plasmid which can be mobilized by R-factors such as RP4.

TABLE I

R-factor mediated chromosome transfer in *Alcaligenes eutrophus* ATCC 17707. Mutant strains were isolated after ethyl methane sulfonate mutagenesis, and crosses performed by filter-mating as described previously (19). Rates of spontaneous reversion to prototrophy by the mutants used was $< 5.10^{-8}$.

Donor	Recipient	Frequency of R-factor transfer	Selected Phenotype	Frequency of Chromosome Transfer (per recipient)
AE7-66/R906 (Leu^-)	AE7-52	8×10^{-1}	Leu^+Met^+	1.1×10^{-4}
AE7-66/R906	AE7-68 (His^-)	6×10^{-1}	Leu^+His^+	4×10^{-5}
AE7-66/R906	AE7-72 (Cys^-)	6×10^{-1}	Leu^+Cys^+	6×10^{-5}
AE7-80/R906 (Phe^-)	AE7-66	6×10^{-1}	Phe^+Leu^+	2×10^{-5}

Chromosome transfer mediated by a natural conjugation system has recently been reported for the nitrogen-fixing hydrogen bacterium (Xanthobacter autotrophicus) (18). We screened several different strains of hydrogen bacteria for transfer of RP4 and other broad host range R-factors (19). Most were rather poor R-factor recipients. An exception was A. eutrophus ATCC 17707 which is both a very good recipient and donor of P1 group R-factors. Both RP4 and R68.45 mediated transfer of several chromosomal markers at a frequency of about 5×10^{-7} in this strain (19). Subsequent experiments with R906, a broad host range R-factor determining sulfathiazole resistance (20), gave much higher frequencies of chromosome transfer (Table I). Several genetic markers were transferred at frequencies of 10^{-5} to 10^{-4}. An important future goal is to develop a genetic linkage map for this organism to serve as a framework for manipulating key genes. A large number of mutants derived from A. eutrophus ATCC 17707, both auxotrophs and autotrophic minus, have now been isolated and partially characterized (unpub.). Many of these mutations have pleiotrophic regulatory effects. Analysis of such mutants can give a better understanding of the mechanism of gene expression in H_2 bacteria which is of great importance for construction of energy efficient and product overproducing strains.

The broad host range DNA cloning vehicle pRK290 (21) can be transferred from Escherichia coli to A. eutrophus at very high frequency (10^{-1} to 10^{0})(19). A genome library of A. eutrophus will be constructed, and cloned genes identified by their ability to complement genetic lesions in specific A. eutrophus mutants. Use of pRK290 will also allow introduction of foreign genes into A. eutrophus.

In conclusion, our knowledge of the H_2 bacteria is advancing rapidly, and both conventional genetic techniques as well as recombinant DNA technology can now be applied to these organisms. This can provide the foundation for genetic engineering of H_2 bacteria for new industrial fermentations.

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* The presentation of R.C. Valentine has been formalized into the preceeding manuscript, at the request of the author and his colleagues, and illustration with references have been prepared as well.

Robert Rabson

When somebody from a federal granting agency comes to a meeting like this, it's with the expectation of hearing about research priorities, not providing them. Nevertheless, for a few minutes, I'd like to relate to you what may be perceived as some of the long-term research needs in areas relating to biological conversion of biomass to fuels and chemicals. I would caution you that there is no coverage of some of the nearer term research needs which also have major importance.

It's always difficult to discuss long-term research needs because of our inability to predict scientific discoveries which can drastically alter directions of thinking. This also represents much of the fascination and challenge of research. It isn't as if this is an operation where an engineering product is being developed or a piece of hardware is the goal. In both of these cases there, too, is a challenge, but the objective is more clearly defined. In attempting to understand complex biological phenomena the research approaches are seldom direct and easy. Many pieces of a large puzzle must come together before understanding is possible. In developing a research program such as the Biological Energy Research program of the Department of Energy for generating fundamental biological information which will serve to provide concepts and support for future biotechnologies relating to energy, we depend a great deal on the feedback obtained from the scientific community. This feedback comes from people like yourselves who are in universities, in industry, and in other institutions. The mechanisms for obtaining feedback include symposia, such as this, workshops in highly focused topic areas, and other communications to ascertain research needs.

There are two major aspects of the Biological Energy Research program. One has to do with the plant sciences and refers to information which Ray Valentine alluded to earlier, namely, on biomass productivity. The second aspect of the program concerns microbiological research dealing with conversions to fuels or chemicals using microbial systems. In both cases the objective is the support of research which generates fundamental information about plants and microorganisms as related to energy questions. May I refer you to a more detailed discussion of research needs in these areas (Rabson,

R., Rogers, P., (1981) The Role of Fundamental Biological Research in Developing Future Biomass Technologies, Biomass, in press).

Regarding the question of research needs relating to the bioconversion of biomass to chemicals, a first consideration is the resource base. The most abundant substrates that will be available are the biopolymers, cellulose, hemicelluloses, and lignins. Although there are chemical techniques such as acid hydrolyses that are available for the conversion of certain of these biopolymers to fermentation substrates, there is yet a good deal of work that must be done in understanding microbial degradation of these biopolymers. This is needed for devising a more economical scheme for saccharification of cellulosic materials to make the use of these materials even more attractive. Thus, it seems that one of the higher priority items should be to gain a much better understanding of how microorganisms degrade these materials as well as the activity of the particular enzymes in attacking the substrate. In general, there is little known of the genetics of any of the cellulose degrading microorganisms such as Trichoderma. The fact that cellulase is really a complex of enzymes makes the problem all the more intriguing. The genetic relationships resulting in integration of synthesis of the component enzymes of the complex are not known. Also, it is only beginning to be understood how the enzymes of the complex are regulated and coordinated biochemically. Of particular interest is that the primary substrate cellulose is insoluble. The spacial relationships of enzymatic attack on such a substrate should prove of considerable interest. Furthermore, there is a completely different array of problems in terms of understanding microbial breakdown of the lignins, a group of substructures for potential exploitation.

Recently, there has been great enthusiasm and controversy generated about the use of ethanol as a fuel. Although the ethanol fermentation is the best known fermentation of any there are still many intriguing problems associated with it. For example, in an understanding of the ethanol tolerance of the fermenting organism, this might be used in the future. Likewise, if we knew more about pathways which provide a broadening of the range of substrates for ethanol fermentations, that would be helpful. In particular, information about the fermentation of pentoses to ethanol is now beginning to attract more attention. This kind of information also offers opportunities for genetic engineering of organisms which could result in the utilization of a wider substrate range, as, for example, the hydrolysis products of hemicelluloses.

Advanced concepts for bioreactors of the future include the attachment of microorganisms or ultimately enzymes to inert matrices to carry out conversions by passage of substrate solutions through the reactor bed. Anothe area of interest in support of such technologies, therefore, is cell surface phenomena to better

understand attachment. Still another area concerns conversions by thermophilic organisms. The prospects of using such organisms to ferment materials to alcohols, solvents and acids as well as in the degradation of biopolymers needs study. The phenomenon of thermophily in itself is a fascinating and critical topic. The advantages of thermophilic conversions include enhanced reaction rates as well as minimization of potential contaminations. There are fermentations which have been used commercially for many years such as the butanol fermentation. However our understanding of the metabolic regulation of this and other conversions is less than adequate as is how production is integrated with various growth stages. As the tools for genetic manipulation continue to become more available it is critical that there be parallel advances in information concerning the physiology and biochemistry of the microorganisms which are to be the target organisms for genetic manipulation.

A good example of an area which is greatly in need of work is methanogenesis. It is a spontaneous process, like photosynthesis, needing no human intervention for mankind to take advantage of it. But when more is known about methanogenesis it might be possible to improve the efficiency of the process significantly. We still know the identity and characteristics of only a few of the organisms that carry out methanogenesis. We know precious little about the interactions among the organisms that are involved in anaerobic digestion that produces methane. When it comes to the genetics of methanogenic organisms it is still essentially an unknown area and this is true to a large extent of a lot of other obligate anaerobic microorganisms. The genetic base needs to be spread somewhat wider to encompass organisms that may be anticipated for use in biotechnologies. By the same token, the powerful genetic techniques such as are being discussed in this meeting provide numerous shortcuts for gaining genetic knowledge rapidly in these other groups of organisms.

These few topics mentioned above, which are certainly not all encompassing by themselves, represent a sizeable agenda to put the biotechnologies of the future on a sound base. There is an enormous amount of work that must be done and it is obviously an exciting time that we find ourselves in regard to microbiology, which is at the center of the current biological revolution.

Last night, we heard Don Helinski mention the growing entry of industry into research in recombinant DNA and associated topics. This is occurring collaboratively with the faculty of many universities as well as independently in industrial laboratories. In view of the need for work in these areas, the opportunities for exploitation for the good of society and the probable static level of involvement by the federal research agencies, these developments can only be welcomed.

When federally derived research monies are tight as they are

now, it is especially important that the available funds be destined for work that has greatest scientific impact. This raises a point which is becoming increasingly troubling to some people in the biomedical research granting agencies as well as others. It concerns the question of openness of research information. The situation is not unique to these areas of research, but it is new here in terms of the scale.

Industry invests in research with the expectation of deriving a financial return on that investment. To achieve that return some proprietary information is involved. This is normal operating procedure. The more or less unique aspect of industrial activity in the recombinant DNA area, in contrast to others, is that much of the research is fundamental in character. Distinguishing the type of research supported by commercial vs. tax monies may become difficult. Given the nature of the situation, secrecy in revealing both the focus of the research activity and the results can be anticipated, although some industrial research laboratories have settled on a policy of considerable openness with no apparent deleterious effects. The question is whether in this unique area of development with the potential of substantial overlap of activities between industrial and governmental support, some workable policy of communication can be evolved to maximize progress for mutual benefit. It seems that duplication of effort need not become rampant, nor should the extended delay or suppression in the reporting of results become commonplace. Some considerable attention would seem worth the effort to attempt the formulation to an understanding acceptable to all parties.

Oldrich Sebek

I have been summoned by our Chairman to appear on this panel about an hour ago and hence did not have much time to prepare a well thought-out presentation. I should confess that I am not completely at ease about this assignment. I am not a molecular biologist or genetic engineer but rather a microbiologist, a microbiologist of the older vintage. Throughout my career, I have adhered to the Delft school of microbiology with Professors A. J. Kluyver and C. B. van Niel as its most prominent representatives and examples. In my scientific life, I have gone through several phases of development. During World War II, we were attempting to utilize waste products by biological methods, a trend which is very much in vogue today. We were fermenting sulfite waste liquors, which are waste products in the cellulose and paper manufacture, to butanol and acetone, to riboflavin and fodder yeasts. After the war, I had the good fortune to come to this country. I studied antibiotics under Dr. S. A. Waksman at Rutgers, lipid and pigment biosynthesis under Professor F. F. Nord at Fordham, bacterial oxidation of polyhydric alcohols at Ohio State, and amino acid metabolism with Dr. H. A. Barker at the University of California at Berkeley. At Upjohn, where I have been for a good many years now, I have been involved primarily in the biosynthesis and biotransformation of antibiotics, steriods, and also of β-carotene and prostaglandins. Without realizing it at that time, these topics exemplify the major areas of microbial physiology and biotechnology in the last forty years, namely the classic fermentations, biosyntheses, and biotransformations. During that period of time, I have witnessed many efforts which were directed toward finding and developing new microbial compounds. To this end, two general approaches were taken: 1.) Screening of known and newly isolated microorganisms as potential producers of such compounds, and 2.) Mutating and selecting those with superior properties. By ingenious modifications of these two basically simple methods, thousands of new compounds have been discovered and economic processes developed for the manufacture of many of them: antibiotics, alkaloids, nucleotides, vitamins, steroid hormones, and others. In the future, additional microbial processes will be economically competitive with the traditional chemical processes because of the increasing cost of the raw material for the latter.

Mutation, which played an important role in this development, is essentially a random process. It has the advantage that it does not require an extensive genetic and biochemical background information on the organism of interest in order to be successful. Thus, practically all the high-producing industrial strains have been obtained by this methodology.

Like other investigators, I am excited about the application of genetic manipulation to these problems. 1.) I visualize that this new methodology will be directed initially toward yield improvment of those therapeutically valuable polypeptides which have already been cloned and expressed in _E. coli_ (human growth hormone, interferon, human insulin A and B chains) and later of others of importance in human and animal nutrition and health. This will be done by cloning the rate-limiting enzymes involved in the biosynthesis of these products. Improvement in the efficiency of biotransformations and preparation of useful product analogues (antibiotics, steroid hormones) will be carried out in a similar manner. 2.) Genetic manipulation will allow also the introduction of qualitative changes into new organisms and the construction of strains with new biochemical functions such as the ability to utilize new substrates (cellulose, lignin) for fermentation, food, and energy.

We are living in times of an increasing awareness and concern about the dwindling supplies of petroleum. Hense as an alternative, renewable raw materials as sources of chemicals, pharmaceuticals, and energy should and must be one of our first and foremost priorities. Before this is accomplished, however, a great deal of basic knowledge will be needed and many problems will have to be solved. Thus, for example, since relatively few genes from different microorganisms have been expressed well in _E. coli_, new more suitable host-vector systems (such as _B. subtilis_, yeasts, or actinomycetes) will have to be developed.

As we are witnessing at this Conference, a large amount of basic information is being generated in this swiftly developing field. This information must not remain a mere scientific curiosity. We must take advantage of this knowledge and apply it whenever possible to the solution of problems of practical significance. Or as Saadi expressed it in Gulistan, a book of maxims, in 1258:

> "Whoever acquires knowledge,
> but does not practice it,
> is like one plows a field
> but does not sow it."

Donald Helinski

First of all, I do not think that I can add substantially more to what I presented yesterday evening. Also, you have heard enough from me and what I would like to encourage now is to have the four panelists and the rest of us hear from you. I would like to make a few comments, however, to maybe structure the discussion somewhat and to go on from there. Bob Rabson stressed, and it came out again in this afternoon's session, that "even with this revolutionary new technology, let's not forget that we don't know everything about microorganisms and we don't know everything about metabolic pathways, so let's continue and hopefully expand in terms of our own efforts towards better understanding these pathways and better understanding these microorganisms". Jim Shapiro made a special plea in this direction in terms of intensifying studies on the organisms that are known to be capable of breaking down aliphatic and aromatic hydrocarbon compounds in order to better understand the biochemistry of those pathways and to understand the genetic process better.

I don't think anyone will argue with the great need for a basic understanding of the biochemistry and physiology of microorganisms. In terms of where do we put our resources, whether it is Federal resoucres, private industry resource, or human resources, clearly this is an area that has to be pushed vigorously. That doesn't mean that we have to know everything about these organisms before we proceed towards genetic manipulation. Dr. Coats stated that there were 900 species of Streptomyces that produced antibiotics. Clearly, it is ridiculous to propose that we should move towards understanding everything about those 900 species. We should focus on certain of the organisms that we know something about, that we have an informational base to start from or that have exceptional promise for a particular objective and then take off from there. It is very important for the Department of Energy along with the National Science Foundation and the National Institutes of Health to get more involved in supporting this kind of basic research. On the other hand, I wouldn't like to be left with the feeling that "before we can make any great leaps forward, we've got to invest a lot of resources and a lot of time in terms of an informational base since the recombinant DNA technology has allowed us to carry out genetic manipulations on organisms that we don't know very much about as to their basic genetics, e.g.,

their conjugal transfer systems or the nature of viruses that infect these organisms". We can overcome much of this ignorance with vector systems that are currently available and we can legitimately expect to do things now in an applied way with these systems.

With regard, again, to basic information, one need that was stressed again and again in the presentations today is an understanding of the regulation of gene expression. It was stressed in Paul Lovett's talk this morning and in John Coat's presentation this afternoon. In the discussion of Actinomycetes, we have seen where many of the problems of expression are going to be overcome by inserting the right promoters upstream from that structural gene that you want to see expressed. This calls for a greater understanding of the structure of promoters, the nature of regulatory signals and how these promoters are recognized by the appropriate RNA polymerases in a specific intracellular environment. It is important to determine the specific requirements for gene expression, not only in E. coli but in other organisms of agricultural, industrial, and medical importance as Pseudomonas, the Actinomycetales, yeast, Neurospora, etc. This is basic information, it's valuable information, it's an informational base to start from and scientists must be provided with the financial support for work in these areas. The recombinant DNA technology has, in certain respects, made scientists feel like a community again, whether you are industrial scientists or academic scientists. The kind of information that we are talking about is not only basic, it is very applied in terms of its overall value.

I stressed last night the great progress that has been made in terms of the variety of plasmids and different systems developed. Clearly, however, much more work must be carried out in the development of vehicles for gene cloning. For example, one very important class of vehicles, and I think this was a focal point of another conference that was organized by Alex Hollaender, Ray Valentine, and Bill Rains, is the development of plasmids that provide stress tolerance to a bacterium that is in its natural environment - as an example, to provide tolerance to a soil organism against high salinity conditions, drought conditions and low temperatures or to provide tolerance to a fermentative organism to a particular stress. I think that there is enough information currently available to design, if not the ultimate plasmid or plasmid system that provides this kind of tolerance, at least a first state plasmid in terms of working out certain models and testing these models in approaching this very important problem. In addition, with regard to the fermentation products, the whole area of extra-cellular products and providing the right information in a plasmid vector for pumping particular protein products across the membrane barriers of the cell, either fungal cell or bacterial cell, is both feasible and of great importance with regard to the ease of purification or the isolation of a particular fermentative product. I was also

struck by the comment of Dr. Coats this afternoon with reference to *Streptomyeces* to the effect that "let's not lose sight of the possibility of developing plasmids that carry in genes that make fermentation energetically a lot cheaper in terms of the source of nutrients for growing bacteria or fungi. I think that this was the point that Ray (Valentine) was getting at with his discussion of the hydrogen bioreactor system. We should be thinking in terms of developing plasmid systems that will facilitate the utilization of hydrogen, and CO_2 with regard to carbon and energy source for driving these biosynthetic pathways for the purpose of producing chemicals or specific proteins. In this regard, it would be desirable to develop plasmid systems that are not going to work just for a single particular species, but are going to have a broad host range capability; for example, an *osmo-tolerant* plasmid that may not only work in different *Rhizobium* species but also with other organisms as *Pseudomonas*, *Bacillus*, and so on.

Finally, a major area that is developing is concerned with enzymes that are more thermostable, either operating *in vivo* as Bob Rabson has emphasized, with regard to being able to increase the temperature of the processes and reducing the energy cost in terms of ethanol purification, etc., or with regard to actually using the bacteria as sources of thermostable enzymes for carrying out chemical reactions *in vitro*. This is an approach that certainly has to be treated with some caution. For example, as Jim Shapiro stated earlier, when we are considering the biochemical conversions of aromatic and aliphatic compounds, we may be talking about very complex enzyme systems, including enzymes that are membrane-bound. There certainly are, however, a variety of enzymes that work quite well in the non-complexed state, where the bacteria or fungi are great sources of these enzymes and where we can now begin be genetic engineering to design enzymes that have the right properties for the catalytic conversions of chemicals *in vitro*. As was pointed out in Fred Heffron's talk today, it is now possible to direct mutations at particular sites within a DNA molecule. He touched upon one mechanism that was developed in his laboratory that is very useful. In addition, there are other procedures currently available for site-specific mutagenesis. It may now be possible with these technologies to bring about specific amino acid substitutions that may make an enzyme more thermostable or process a longer half-life in terms of use *in vitro* for carrying out particular catalytic conversions. It may also be possible to begin to develop enzymes that are better able to work in a hydrophobic environment which is going to be necessary for many kinds of chemical conversions. We clearly are in a revolutionary era with regard to the availability of techniques and opportunities. We shouldn't lose sight of this and we should push this branch of science vigorously.

Discussion

Q. STODDARD: My comments have to do mainly with energy distribution. As we all know, most of the energy consumed in this country is produced and distributed on a centralized type of a scale, and I would hope that we don't allow our familiarity with this pattern in energy distribution to influence the directions that we may take in the distribution of biologically produced energy. Two main points should be considered; one, economical and the other, biological. It would seem to me that the pros and cons of centralized energy production and distribution have to do largely with economical concerns of distribution of the energy on the one hand, and the building of facilities on the other hand. Do we build a lot of small energy production facilities on a very local scale? Or do we build large ones and pursue that route? The main differences in those two strategies of energy distribution may boil down to generating energy for large urban centers or generating energy for production and consumption on farms and in rural areas.

Another area on which I wanted to comment had to do with feasibility of using highly engineered organisms on a small scale. If we have methane generators or ethanol generators, on rural settings where they are being used to power farm machinery or any part of small scale energy production, is it feasible to use highly engineered organisms which may have very specific culture requirements on that small scale? Also, doesn't that preclude the use of engineered organisms on a large scale because of the problems of controlling conditions?

A. VALENTINE: I'll try to be brief as I think there are a number of other people who would like to make some comments. First, regarding the biomass, the very nature of the biomass is quite diffuse and I think that almost automatically you are likely to have more dispersal of energy production or chemical production. That is, I doubt that you are going to be shipping corn cobs to California. I think you are going to have more regional planning, more state by state, more region by region planning of the distribution and collection of the biomass. So, you are likely to see decentralization. In California, with a lot of big diesel tractors, the farmers are quite concerned about the price of diesel fuel. They keep coming to the University and asking, well, we can't burn ethanol (gasohol) in a diesel engine. But we can burn a butanol mixture.

What can you do about that as a genetic engineer? Well, several of them tried the butanol fermentation recently on a small scale. They succeeded in making butyric acid, large amounts of it. So, if somebody could simply engineer a system, perhaps involving only a few mutations, it might be possible to block the ability of that organism to make butyric acid. I think that using genetic engineering prudently, we can come up with organisms that are far more failsafe than now. I don't recommend anyone in the audience trying to run the butanol fermentation right now on the farm. The genetically engineered organism would really be great for that.

A. RABSON: A number of studies have been conducted on the energy and economic costs of biomass conversion plants. The concensus of such studies is that those costs prohibit massive central facilities which would be used to process materials collected over a very wide radius. Generally speaking, a collecting radius no greater than one hundred kilometers would probably be the limit for most biomass.

A. HESPELL: In response to the on-the-farm operation, there are several organisms currently available for methane formation from farm residues that are economically feasible right now and are in operation in Pennsylvania. Another concept has been lacking throughout most of the talks so far. Most people are talking in terms of a single organism and manipulating that organism either genetically or environmentally. Now environmental control can involve hydrogen, for instance, partial pressure, and regulation of electron carrying nucleotides. Controlling whether you make acetate or ethanol is purely an environmental control. Another type of control along these lines is the concept of coupling two or more organisms for a multi-stage fermentation which occurs in a single vat. For example, coupling a hydrogen-producing organism with a hydrogen-utilizing organism. There are many other possibilities, for instance, coupling an organism which makes acetoin. If you do not wish the lactic acid and convert it to another product, propionate.

Q. WALTON: I would like to pick up on a more specific question that I think Dr. Helinski mentioned, which certainly is involved in adding to the concept of modifying enzymes to be more temperature-stable or perhaps pH-stable. Can you follow this up and give us some clues as to which direction one should go, or does one have to use a completely random approach which is not really logical at this time?

A. HELINSKI: I would like to make two comments. First of all, I am very much impressed by the kind of information that various groups have been obtaining with regard to the three dimensional structure of specific proteins or specific model systems. What is necessary in an increased effort with regard to these kinds of studies in order for us to obtain a better understanding of the three-dimensional

structure of an enzyme, including information as to possibilities for cross-linking polypeptide chains by the introduction of cysteine residues with the possibility of obtaining a more thermostable protein. This is one area that currently demands a lot more attention. It brings in not only protein biochemistry and physical chemistry but also recombinant DNA technology in order to introduce specific mutations in the gene encoding for that protein. Clearly we are at the model building stage in terms of this particular approach.

I am not willing to write off the random mutation approach for the following reason. If one has an enzyme that is produced by an organism such as *Pseudomonas aeruginosa*, the gene specifying that enzyme may be one of some 5,000 genes in that organism. The traditional approach has been to mutagenize that organism in order to introduce a number of mutations and then screen for or select the organism that produces an enzyme with modified properties or more desirable properties for whatever purposes one is introducing the mutation. What the recombinant DNA technology does for us is that it allows us to isolate and carry out mutagenesis on a specific gene and not on the whole organism. We are painfully aware that when we carry out mutagenesis on the whole organism that even though we may introduce a mutation that is potentially useful in the gene we are interested in, we are also likely to introduce a number of other mutations that now may make that organism less desirable for fermentation. With recombinant DNA technology, if we have an enzyme that consists of 300 amino acids requiring 900 nucleotide base pairs for coding, we can introduce mutations within this 900 bp sequence and, therefore, greatly increase the probability that the mutational event will bring about a change only in the properties of the enzyme or protein of our interest. It makes it more reasonable, then, to go ahead and screen for those particular mutational events that bring about the desired change in the properties of the enzyme of our interest.

HOTCHKISS: One hears quite a lot about looking for desirable mutations. I was beginning to worry about the undesirable mutations after you have selected a product and begun to produce it industrially. We know of the substantial fidelity of DNA copying, but we also expect a certain number of spontaneous mutations during the course of a long propagation as used in preparation of a batch of product. It seems to me it could be quite disastrous if in an evolutionary sense you can only reward your organism for making Chlorophenicol resistance or some plasmid marker and you cannot create the environment where it pays the organisms to make insulin or some brain hormone, let's say. Your stockholders would be glad to pay it but they don't know how, and I am wondering if you are not in some danger of developing near mutant copies, near analogs possibly inhibitory or toxic of the desired products -- about which there is no prior knowledge or experience. Mutation along such

lines would be unrestricted, except with constant monitoring for unknown products and extremely frequent subcloning. I wonder how much thinking has been going on so far about this kind of evolution. To an observer, there seem to be so many plasmids turning up so fast, I have the impression that each one can only have been worked with for about twenty minutes!

I am not speaking about the moral issue of irresponsibly ignoring consequences; I am asking, isn't there a practical likelihood that ethical manufacturers may slowly or abruptly be faced with undesirable impurities and loss of production? In some support of my concern, let me simply remind you how often we have heard the finding that this or that gene already became subtly mutated, or unexpectedly modified, or only weakly expressed when inserted in a new environment.

EVELEIGH: I have two points in regard to approaches for using biomass, without being the Devil's Advocate. I would hate to see Dr. Valentine's suggestion of effort being put into industrial catalytic conversion of biomass to hydrogen, in order that 'hydrogen bacteria' can be applied. This industrial conversion process is inefficient in terms of percent yield. In contrast, processes based on biomass substrates have to be efficient, as the process is sensitive to the high cost of the substrate. This is not to say 'hydrogen' bacteria or methylotrophs should not be developed for large scale availability if inexpensive substrates are available from coal, i.e., syn-gas and its conversion to methanol. The advantage of biological conversion of biomass is the high efficiency of conversion and this needs stressing in relation to chemical conversion systems.

The second point is that we need to take stock, occasionally, of the potential biomass. It is often referred that biomass will solve the energy crisis in the United States. It will not. With intensive effort, biomass is predicted to supply 10% of U.S. energy needs (perhaps 10 quads/year). A philosophical problem is thus, to what use should biomass be put as a substrate? As a source of alternate energy materials (e.g., ethanol as a liquid transportation fuel) or for production of antibiotics, steroids, etc.? A savings of energy is accomplished in the latter case and also addresses producing a high priced product. The problem there is, should we devote our university research efforts to commercial processes or academically intriguing ones? There is no easy answer.

HELINSKI: At the risk of sounding idealistic, I think most of us are thinking of profit as profit for society and not profit for individuals, and I think that is what we should be addressing in this particular conference. On the issue of energy, I certainly did not come to this conference with the idea that we are going to

gain independence from foreign sources of oil on the basis of manipulating microorganisms. While I am optimistic with regard to the value of these techniques, I am not prepared to advance the notion that they are going to solve all of society's problems. I do feel, however, that we can make positive and dramatic contributions to a number of these problems. Rollin, I wasn't very sure whether you were getting to the pcint of undesirable mutations from a biohazard standpoint. Many of us in this room have gone through the whole process during the past six years of these kinds of considerations. I must say that as someone who has been working in the field of plasmids, that the natural biosphere is far outproducing us in terms of nucleotide sequence changes and structural changes in plasmids. Even if we totally mobilized the bright, young people in this country to have them work on constructing plasmids we would not keep up with what is naturally occuring in our environment on a daily basis or have much impact on the total number of spontaneous genetic changes that are occuring.

CHASE: I have only some main thoughts to go over, in our discussion here and the second thought, which will be question, I can throw out to everyone. One is on thermophily. I agree with an earlier statement suggesting that we have very little knowledge as to the basis of what makes one protein more thermostable than another. I think I recall a paper some years ago where people examined amylases in many strains of essentially the same bacterial species and they were concluding much about what the difference was, perhaps it was more free in the more thermophilic strains. I think perhaps a bigger problem is that if we are talking about making a whole organism thermophilic, we have to make every enzyme within the organism thermostable, and I don't know whether that is a feasible project for even the most dedicated direct mutationist. I think that we are talking about making two or three extracellular enzymes thermostable. We are talking about thermostable celluloses plus the thermostable amylases.

The other thought crossing my mind is that we have a great deal of discussion on how to make chemicals which are known to be produced in low yields by organisms of one sort or another. Is anybody devoting any thought as to how to approach making chemicals which are not now biological products? Let me give an example. One of the major oil companies routinely puts 7½% T-butanol into its gasoline. This has very desirable anti-knock properties and in effect, they are virtually making a gasohol product right there except that T-butanol, I am sure, is also petroleum-derived. Is anyone thinking or perhaps somebody has already thought, of how to make that compound biologically?

A. HELINSKI: I don't want to comment on your second question because it is totally out of my area. But, with regard to your

first comment, I think what you have raised here is a point that shouldn't be overlooked. We are certainly not going to take a single organism or a half dozen organisms and convey to these organisms the genetic traits that are going to carry out all of the possible processes that we wish to engineer into microorganisms. Quite clearly we have to start with a microorganism that is near our final product. There are a great number of bacteria that have evolved and found their niche in our biosphere and possess a number of desirable properties that we do not even know exist, or where we haven't really made the effort to search for these kinds of organisms. If we are talking about thermophilic organisms that can be used to produce a thermostable enzyme, there are naturally occuring thermophilic *Pseudomonas*, of course. We may wish to introduce a mutant gene that produces a thermostable enzyme into this organism. This clearly is a more feasible approach than starting with a mesophilic organism and attempting to introduce into this organism, from a genetic standpoint, thermophilic growth properties in addition to a specific thermophilic enzyme. Once again, on the positive side, we don't have to work up a conjugation system or find a virus to infect that particular organism to introduce this gene. With the recombinant DNA technology, we can use existing cloning vehicles to move these genes from one organism to another.

VALENTINE: We had a symposium a couple of years ago dealing with osmoregulation. It was clear at the time of that meeting that there was a great deal of unity between the various types of environmental stresses, all the way from thermal to osmotic to cold and chill. In fact, it turned out during the course of the meeting that many of the man-made temperature-sensitive proteins, while inactive at high temperature, at normal osmotic strength, are activated by simply raising the osmotic strength of the medium. In other words, all of these defective mutations became now tolerant to temperature in high osmotic conditions. You might gain five degrees in maximum by modifying the growth temperature and osmotic strength of the medium. Clearly, there are some creative, new approaches to the thermophilic problem. We don't know much about it but we can't fall back on the old dogma. We do know a little bit and if we start with that, I think we can slowly build a base for the mechanism of thermophily. It is not an unsolvable problem and as Dr. Helinski has stated, I think recombinant DNA has a lot to offer.

A few years ago people were talking about osmophilia and how insoluble it was. An osmoregulation gene has now been cloned. I think one of these days you will see similar progress in the thermophily area.

There is utterly no question that as you go into the higher scale manufacturing of fermentation chemicals - above the test tube

tube level, there is a tremendous selective pressure against over-producing mutants (genetically engineered or otherwise) and stabilizing these cultures will be difficult. Of course, the problems of stability of genetically engineered organisms is difficult, but I believe the technology is such now that we can solve the problems.

GUNSALUS: A couple of questions have been recurring and bothersome to me. First, adequate strategies exist for the isolation from Nature of organisms resistant to reagents or those able to use selected substrates. In contrast, I'm unaware of strategies for "fishing out" from Nature organisms that will perform a general function or make a given compound, e.g., a substrate if available, abundant, or inexpensive, or all three. How is this "feedstock" converted to a useful and/or saleable commodity, or manufacturing intermediate?

Second, what consideration should one devote to thermophiles? I presume the objective is to avoid other organisms, i.e., "contaminants", and to obtain enhanced rate process. To me the handicap is in the investigators imagining properties Nature has selected for the conditions suitable to the preferred organism. These do exist in ecological niches. These, I believe, are the places Don Helinski indicated where one should fish. One strategy is to select an experimental situation adaptable to upscaled processing, the assurance that the product can be produced.

What are you really talking about when you suggest getting organisms more thermophilic, that is, to grow rapidly and produce products at higher temperatures? What do you know about the cell and the components in a structural way, particularly in obligate thermophiles? Are the products (enzyme, etc.), once isolated, more temperature stable? If a thermophile is grown at, say, 65°C, are the proteins when isolated more stable than from the same strain grown at a lower temperature, e.g., 40°C±? There may be natural modifications, I would presume in the hydrophobic regions around the catalytic site rather than the modification of the protein primary sequences. This, of course, is an assertion. That is, what are the structural differences between proteins of mesophilic and thermophilic strains?

Another related question is the time required to find or select cells more resistant or with more activity. Do you have a clear picture of the fraction of cells in a population that carry the phenotype which is being sought, and in mind a selective procedure to obtain the same? In what way do you wish to modify the gene pools? Is the end product of the selection, a process, or a genetic stock? What are the rules in Nature for selection and survival, particularly for thriving in a very complex environment, not only at any given time, but the sequence of events when new material is added -- a discontinuous environmental continuation

with abrupt alterations and many graduations. How much can one modify a protein and still maintain, or better yet, improve activity? What message do we get by looking at Nature's current, or so called "normal" structures? What residues were selected, i.e., those that are retained, or should I say, modified? What are the criteria when you focus on a protein, the one you wish to modify? The question is modify to be or do what? One knows in a cell the concert of processes and levels of structures compete for maintenance. The question becomes into what is this array to be modified?

Diffuse and poorly formed as these recurrent questions are, on listening to the statements of the objectives and procedures in this wool-gathering session, I feel they impinge on the essence of biology, our imaginations, and discoveries of Nature's mechanisms concerning the accommodation of organisms in environmental niches and the constantly changing facets in the environment, that is, the evolutionary processes, if you wish. Perhaps what we are seeking is to aid Nature's race with a quantum jump.

It occurs to me, in contrast to some earlier remarks here, in the most developed research areas in industry, the primary function is to solve problems. One is likely to find, particularly in the corporate research laboratories those whose sole function is to solve the fundamental problems related to developments of a new use area for financial return, i.e., build a new industry product intermediate to perform a service in such need.

May I ask, who has brought forward the modifications which we now enjoy, for example, the transmitting of information on fibers instead of copper wire, or replacement of silk by synthetic fiber? Where are the concepts being developed? Where is the naivety we speak of as primative or unsophisticated, the growth of awareness given the opportunity to solve or to initiate, as the case may be? Where are these coming from, if not mainly or substantially the universities? Might I say, come on, who do you think we are kidding?

CHASE: Just one more thought, if we are still talking about thermophily, perhaps a better approach would be to take a thermophilic organism and mutate one of its existing enzymes until it does something quite different. I think we are probably closer to being better able to design an approach that way than we are to being able to design it to thermophily. And certainly we've got the greater simplicity for working on only one protein or a few proteins rather than all of the proteins in an organism. Is there anybody working on that yet?

WOOD: Well, that problem is separate and looks like E.C.C. Lin tried to approach a very subtle aspect of this problem and has set up the model. He merely tried to change the Km of an enzyme for a

substrate that didn't bind very well. It happered to be ribitol dehydrogenase binding xylitol as a matter of detail and he claimed to have obtained better binding. But, this has by no means been duplicated by others nor has this whole line of approach been followed by anyone. It has not been easy to change the quantitation of kinetic parameters nor has it been easy to change specificity, let alone go to a different reaction mechanism. Now there may be a few examples of catalyzing similar reactions, apparently different reactions, but having a common mechanism, but I don't think that's quite as purposeful as one might try based on that kind of work.

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