Genetic Manipulation of Antibiotic-Producing Microorganisms

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Summary. The application of directed selection techniques and genetic engineering methods for manipulation of antibiotic-producing microorganisms is generating a new era in industrial microbiology. Modern methods, based on advances in the knowledge of the biosynthetic pathways and regulatory mechanisms involved in the induction and repression of genes involved in antibiotic synthesis, provide a means of increasing antibiotic activity. Hence, recombinant DNA and protoplast fusion methods are used to alter the genetics of antibiotic producers in a semirandom fashion for the development of novel hybrid antibiotics. Directed mutation and selection, protoplast fusion, and both semirandom and specific recombinant DNA methods are examples of alternative procedures for manipulating the biosynthetic pathways of microorganisms for strain improvement and for new hybrid antibiotic synthesis.

Manipulation of the biosynthetic pathways for antibiotic synthesis in producer microorganisms has generated clinically important antibacterial and antitumor agents during the past 30 years (*1*). There are now approximately 150 antibiotic compounds on the market. Antibiotics that become commercially important are discovered and further developed by research efforts in microbiology and genetics aimed at screening microorganisms for naturally occurring secondary metabolites, by modifying natural substances chemically to produce semisynthetic derivatives having desirable characteristics, and by improvement of techniques for selecting mutants that produce useful antibiotics in large amounts. Compounds must be both cost- and health-effective in order to become marketable.

Approximately 7000 naturally occurring and more than 30,000 semisynthetically derived antibiotics have been discovered since the early part of this century (*1*). In most cases these compounds were obtained or identified by empirical methods (*2*). There have also been great improvements in the techniques used for increasing antibiotic productivity of particular microorganisms, and these have resulted, primarily, from random approaches. The future of antibiotic screening and strain improvement technology should move away from the empirical, random methods of the past toward the application of scientifically more rigorous approaches. Advances in the understanding of the antibiotic biosynthetic pathways in many organisms (*3*), in the elucidation of regulatory mechanisms related to the induction and repression of genes involved in antibiotic synthesis (*3*), and in the physiology of the relevant microorganisms, make it possible to develop and begin to apply new, nonempirical strategies for both strain improvement and novel antibiotic development. Genetic engineering techniques, including recombinant DNA methods, can facilitate the application of some of these new strategies (*4*, *5*). This article focuses on the biology of antibiotic-producing microorganisms and has three primary objectives

1) To describe current methods for the genetic improvement of organisms leading to the development of strains that produce large quantities of known antibiotics,

2) To describe methods for altering the genetic structure of antibiotic-producing microorganisms in a controlled, but half-random, fashion to facilitate the biological synthesis of new, improved antibiotics, and,

3) To describe strategies for the specific alteration of antibiotic synthesis pathways to create new, slightly modified, strains capable of the biological production of known antibiotics currently manufactured by semisynthetic chemical methods.

Selection Techniques: Production of Antibiotics by Mutation and Selection

Historically, the strategies used for the manipulation and screening of microorganisms to generate strains that produce large amounts of antibiotics of commercial importance have included, primarily, random mutation and selection (*1*, *2*). These methods, although successful in some cases (*4*), are based on empirical experience and have, in general, a moderate probability of success. They are "hit or miss" methods that require brute force effort, persistence, and skill in the art of microbiology. These older technologies were developed at a time when knowledge of the genetics and biochemistry of antibiotic-producer organisms was very limited.

A number of the classical methods have, in recent years, been replaced by more "rational" (that is, directed) selection techniques (*6*), such as (i) direct colony selection following overlay bioassay, (ii) selection for mutants resistant to toxic precursors or to the toxicity of end product, (iii) use of analog resistant mutants that overproduce rate-limiting biosynthetic intermediates, (iv) selection for mutants resistant to metallic ions that complex antibiotic molecules, (v) selection of auxotrophic strains deficient for biosynthetic intermediates followed by prototrophic reversion, (vi) selection for improved strains after protoplast fusion, and (vii) use of antibiotic selection for the isolation of multiple plasmid-copy strains.

These directed procedures can be much more efficient than random screening for the selection of improved antibiotic-producing strains, since each one is based on studied, known parameters that affect the regulation of the biosynthetic pathway of a particular antibiotic in the producer microorganism. They involve the preliminary screening of mutagenized cells before they are used for laboratory (small-scale) fermentation studies as well as for selection strategies based on known or probable biochemical mechanisms (*4*), as indicated below.

The work of Elander *et al.* (*7*) and of Chang and Elander (*6*) on the development of methods for selecting strains of *Acremonium* (*Cephalosporium*) *chrysogenum* that produce high yields of the commercially important antibiotic ceph-

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alosporin C illustrates the application of the overlay bioassay procedure. Prior to the development of this method, mutagenized *A. chrysogenum* colonies were picked from agar plates, at random, and were grown in shake flasks for drug assays. In the overlay assay previously mutagenized *A. chrysogenum* colonies are plated onto agar, and the plates are incubated; the antibiotic activities of the resulting cells were assayed by allowing a second, antibiotic-sensitive organism to grow over the original colonies. Colonies that produce antibiotic can be observed by the appearance of zones of inhibition. An index of potency (PI), defined as the ratio of the diameter of the zone of inhibition to the diameter of the antibiotic-producing colony, can then be determined. Colonies are picked for further analysis based on an arbitrary PI; for example, all colonies having a PI greater than 4. This procedure can increase the probability of identifying high producer strains. Chang and Elander (*6*) demonstrate that this method can improve the efficiency of screening methods by a factor of nearly 10. This basic concept can be used to further increase the probability of isolating desirable strains by manipulation of the medium and growth conditions for both the producer and tester strains.

Selection for resistance to toxicity of end product. The β-lactam containing antibiotics include a large number of different compounds that are produced as secondary metabolites (*1*) by both prokaryotic and eukaryotic microorganisms. The range and characteristics of the many β-lactam–producing species have been described (*4*). One of the more novel β-lactam compounds is nocardicin A which is produced by the actinomycete *Nocardia uniformis* (*8*). The substance is the major active component of a series of related secondary metabolites. The compound has in vivo activity in mice infected with strains of *Pseudomonas aeruginosa*. The discovery of this cell wall–active antibiotic from *N. uniformis* was detected with the use of a β-lactam supersensitive mutant of *Escherichia coli* (*8*). The biosynthetic precursors of the compound appear to be L-p-hydroxyphenylglycine, L-serine, and L-homoserine; these precursors are not the same as those precursors for the majority of the clinically important β-lactams.

Two major problems were encountered during attempts at selection for improved strains. First, it was found that *N. uniformis* is itself sensitive to a range of β-lactam antibiotics, including nocardicin A. Growth of the microorganisms

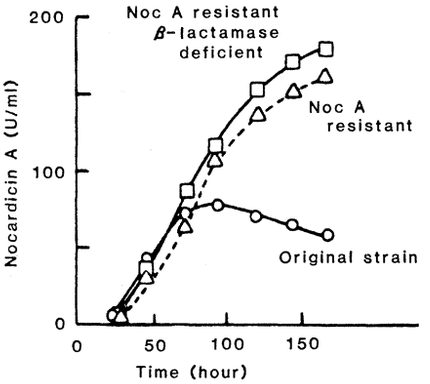

Fig. 1. Improved nocardicin A production by a β-lactamase–deficient and nocardicin A–resistant mutant of *Nocardia uniformis*.

could be inhibited by the addition, at a concentration of 2 milligrams per milliliter, of the drug at different times in the fermentation, generating fragmented and lysed cells. This problem was addressed by selecting for strains that were mutagenized by either ultraviolet radiation or with nitrosoguanidine and that would survive on agar plates containing varying concentrations of nocardicin A.

The second problem developed as attempts to solve the first proceeded. A number of the mutants resistant to nocardicin A produced significant amounts of β-lactamase that destroyed nocardicin A and other β-lactam antibiotics. The β-lactamase was secreted into the culture medium. It became, therefore, necessary to devise a selection for the subset of mutants resistant to nocardicin A that were β-lactamase negative. Mutagen-treated *N. uniformis* spores were plated onto an agar medium. Soft agar, containing a dilute culture of *Staphylococcus aureus* plus penicillin G (varying concentrations) was overlayed, and the plates were incubated at 37°C for 18 hours. Growth zones of *S. aureus* were observed around colonies that produced β-lactamase, which in turn hydrolyzed the penicillin G in the medium. The β-lactamase negative mutants were selected as colonies showing no growth around them. Stable β-lactamase mutants of *N. utilis* which did not revert to become β-lactamase positive, were then screened for resistance to nocardicin A as described above. The frequency of mutants superior to parent strains in producing nocardicin A was higher when mutagen-treated spores were selected from media containing nocardicin A at concentrations equal to or higher than that which inhibited growth of the parent (Fig. 1).

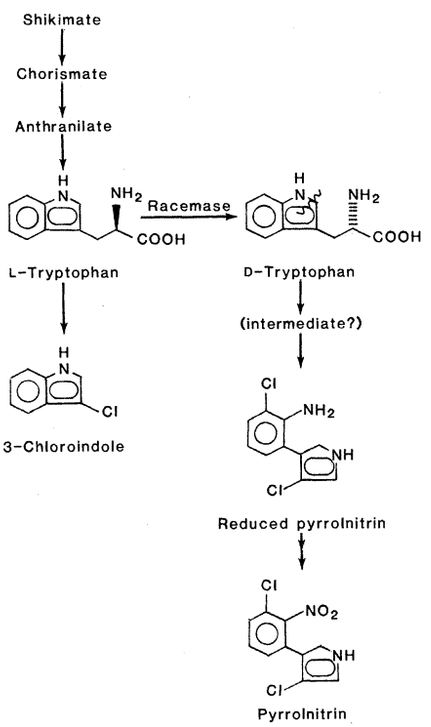

Fig. 2. The biosynthetic pathway of pyrrolnitrin synthesis in *Pseudomonas fluorescens*, illustrating the role of D-tryptophan as precursor.

Thus, selection for two traits, a resistance to toxic levels of end product and the inability to produce an antibiotic degrading enzyme, greatly improved the productivity of the desired antibiotic, in this case nocardicin A.

Selection for analog resistant mutants. Mutants resistant to amino acid analogs have been used successfully for the overproduction of antibiotics having amino acids as precursors. One example, reported by Elander (*9*), was the selection of higher pyrrolnitrin-producing strains of *Pseudomonas fluorescens*. Pyrrolnitrin (Fig. 2) is an important antifungal agent for which D-tryptophan is a precursor. Selection methods that included either fluoro- or methyltryptophan in the medium generated a set of mutants that overproduced D-tryptophan, which increased the productivity of pyrrolnitrin more than threefold and, in addition, eliminated the need for adding D-tryptophan to the medium for maximal pyrrolnitrin formation. Other examples where this strategy has proved successful include the selection for mutants of *Penicillium* and *Acremonium* that overproduce the very important β-lactam antibiotics of the penicillin and cephalosporin classes, respectively. The biosynthetic pathways for both penicillin and cephalosporin compounds involve the amino acids α-aminoadipic acid, cysteine, and valine as precursors (Fig. 3). A series of analogs of these amino acids (Table 1) was used to select for strains with improved cephalosporin C produc-

tivity (*9*). This approach increased the probability of finding such strains. In addition, methionine analogs were used in separate selection experiments. Although methionine is not a precursor for cephalosporin C biosynthesis, it is a required nutrient for optimal fermentation of the microorganism. Strains were selected that overproduce cephalosporin C, although knowledge of the mechanism remains limited.

Selection for resistance to metal ions. Ions of heavy metals such as mercuric ions (Hg^{2+}), cupric ions (Cu^{2+}), and related organometallic ions are known to form complexes with β-lactam molecules at high concentrations. It is possible that many of the mutants which become resistant to these metallic ions may do so by overproducing β-lactam antibiotics as a means of detoxification, thus preventing metal binding and possible interference with SH-containing compounds that may be substrate intermediates, or binding at active sites of metal ion requiring enzymes. For example, mutants have been isolated for resistance to phenylmercuric acetate (PMA) for improved cephamycin precursor production in *Streptomyces lipmanii* (*10*). Mutants resistant to inhibitory concentrations of Cu^{2+}, PMA, chromate, Mn^{2+}, and Hg^{2+} have been isolated and tested for cephalosporin C production in shake flasks (*11*). The results show that the frequency of superior isolates among mutants resistant to mercuric chloride is higher than for isolates selected at random among survivors of ultraviolet treatment. Isolation of mutants resistant to organometallic compounds has been used for selection of specific types of nutritional mutants. A new class of methionine auxotrophs in *Saccharomyces cerevisiae* has been obtained by isolating methylmercury resistant mutants (*12*).

Selection of auxotrophs deficient for biosynthetic intermediates followed by prototrophic reversion. The use of auxotrophs for overproduction of amino acids involved as precursors in antibiotic production has been successfully applied (*13*). The operative principle in this approach is to block, by mutation, one part of a branched biosynthetic pathway so that metabolites flow through another branch. Overproduction of a metabolic precursor, and, subsequently, the antibiotic itself, can occur. Work with *S. lipmanii* (*10*) where certain auxotrophs of pyruvate and aspartate metabolism produced increased titers of cephamycin, and by Treichler *et al.* (*14*) where sulfur metabolic auxotrophs resulted in the improved fermentation of cephalosporin C in *A. chrysogenum*, are two well-documented examples illustrating the strength of this approach. Our work has resulted in the isolation of a limited number of *A. chrysogenum* auxotrophs to various nutrients (see Table 2) that are either inferior or normal producers of cephalosporin C. When reverted to prototrophy, a number of these become superior drug producers, having been derived from either high or low producing auxotrophs.

Table 1. Screening of mutants resistant to various amino acid analogs.

Analog	Antagonized amino acid	Isolates		
		Resistant tested (No.)	Superior retained	
			(No.)	(%)
Selenocysteine	Cysteine	45	0	0
Allylglycine	Cysteine	25	0	0
Norvaline	Valine	27	0	0
DL-α-aminobutyric acid	Valine	151	1	0.66
S-2-aminoethyl-L-cysteine	Lysine	16	1	6.2
Selenomethionine	Methionine	153	8	5.2
Trifluoromethionine	Methionine	55	2	3.6
Selenoethionine	Methionine	87	6	7
α-Methylserine	Serine	25	1	4
Trifluoroleucine	Leucine	35	1	3

Selection for improved strains after protoplast fusion. Protoplast fusion technology can be used in combination with selection techniques previously described to generate industrial microorganisms having desirable properties. In general, the biochemical mechanisms that result in stable hybrid strains as a result of protoplast fusion and subsequent regeneration are not understood. In spite of this, the technique provides an effective method for the intermixing of genes of two (or more) strains having a set of characteristics that, if combined into a hybrid organism, may improve antibiotic productivity. The work of Okanishi *et al.* (*15*), Ferenczy *et al.* (*16*), and others in protoplast formation, fusion, and regeneration has accelerated the development of the techniques used in gene manipulation in *Streptomyces* and other microorganisms. Complementary auxotrophic strains of a given organism are used in this method. Protoplasts are prepared by treating cells with various lytic enzymes. The presence of an osmotic stabilizer is essential to provide osmotic support for the protoplasts, and fusion is enhanced by the addition of polyethylene glycol and Mg^{2+}. The techniques for formation, fusion, and regeneration are well described (*17, 18*). Both intra- and inter-species fusions have been reported (*17*).

Significant results in strain improve-

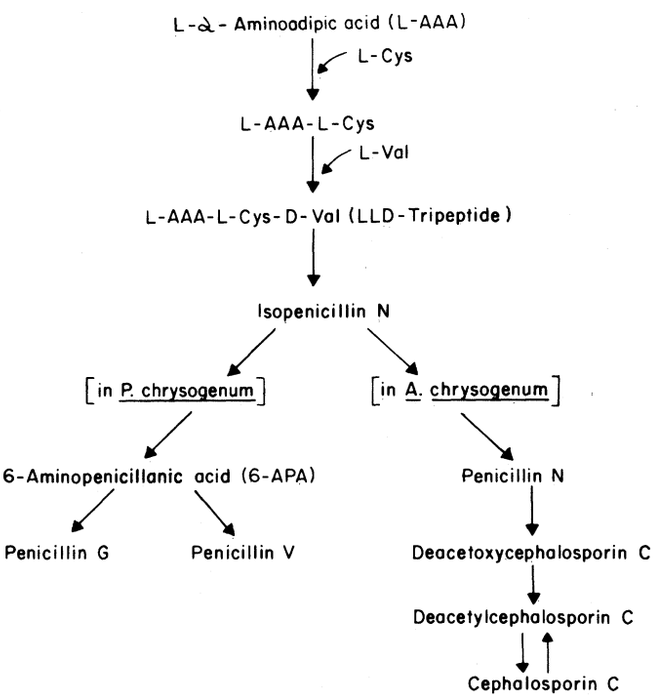

Fig. 3. Biosynthetic pathways for penicillin and cephalosporin production illustrating the various amino acid precursors.

Table 2. Cephalosporin C production by auxotrophs and some of their revertants.

Strain No.	Nutritional requirement	Cephalosporin C titer (% of control)	Revertants tested (No.)	Superior retained (No.)
A2	Nicotinic acid	10		
A20	Uracil	100		
A47	Serine	110	12	1
A101	Uracil	95		
A103	Thymine	110		
A104	NH_4^+	120	10	1
A114	Riboflavin	90		
A115	Riboflavin	50		
A117	Xanthine	110		
A118	Cysteine or methionine	35	25	2
A119	Cysteine or methionine	10	20	0
A120	Xanthine	10		
A125	Lysine	10	5	0
A127	NH_4^+	65		
A128	NH_4^+	70		
Control	None	100		

ment have been obtained by means of protoplast fusion. Hamlyn and Ball (*19*) have, for example, made hybrid strains of nutritional auxotrophs of *A. chrysogenum*, which produce significantly higher amounts of cephalosporin C, and which can produce cephalosporins efficiently from inorganic sulfate. Protoplast fusion has been applied (*17*) for the development of fast-growing strains of *P. chrysogenum*, which produce very little *p*-hydroxypenicillin V. The development of such strains is economically important because contamination of penicillin V parahydroxylated by-product leads to interference with the chemical ring expansion steps of penicillin to cephalosporin. An excellent example of combined rational selection and protoplast fusion procedures has been described (*20*) for improving carbapenem-producing strains of *Streptomyces griseus*. This organism produces the novel carbapenem antibiotics, C-19393 H_2 and S_2 (carpetimycin A and B) and E_5, as well as epithienamycins and olivanic acids. These compounds are classified into two categories, that is, sulfated (C-19393 S_2, MM-4550, MM-13902, and MM-17880) and unsulfated (C-19393 H_2, E_5, epithienamycins A, B, C, and D) carbapenem antibiotics. Studies on the production of these antibiotics in a chemically defined medium show that the production ratio of these two groups of antibiotics is affected by the sulfate concentration in the medium. Mutants unable to produce sulfated carbapenem antibiotics were successfully derived and were found to be sulfate transport–negative mutants. They were isolated as either auxotrophs requiring thiosulfate or cysteine for growth or mutants resistant to selenate. They produce unsulfated carbapenem antibiotics at almost twice the levels of the parental strain C-19393. Converged accumulation of C-19393 H_2 was achieved by selecting for strains resistant to *S*-2-aminoethyl-L-cysteine from a sulfate transport–negative mutant. A second mutant produced C-19393 H_2 as the major component and showed a potency ten times greater than that of the original strain C-19393. The sulfate transport system was reintroduced into sulfate transport–negative mutants from the strain C-19393 through protoplast fusion. Stable genetic recombinants were obtained at high frequency by treating the mixed protoplasts with 40 percent polyethylene glycol. High producers of C-19393 S_2 were found among these recombinants. The above examples indicate that the protoplast fusion approach may have a significant impact on the future of antibiotic strain improvement.

Antibiotic selection for plasmid-containing strains. The role of plasmids in the biosynthesis of antibiotics was initially suggested by Okanishi *et al.* (*21*). Genes carried by plasmids are thought to be involved in resistance to antibiotics, formation of morphological characteristics, and many other phenotypic properties of antibiotic-forming microorganisms (*22*).

Streptomyces kasugaensis produces aureothricin, thiolutin, and kasugamycin. The production of all three antibiotics can be eliminated by the so-called plasmid "curing" treatment. Intensive study of aureothricin biosynthesis (*23*) showed that the structural genes for aureothricin biosynthesis seem to be localized on a chromosome, whereas plasmid genes may be involved in determining the properties of the membrane, such as transport mechanisms, and can affect the cell's ability to accumulate precursors of antibiotic synthesis. Another well-studied model of the interaction of plasmids with antibiotic synthesis is the production of chloramphenicol by *Streptomyces venezuelae* (*24*). It has been found that the structural genes responsible for antibiotic synthesis are usually located on a chromosome, and manipulation of endogenous plasmids may affect the increase in production of chloramphenicol.

The genetics of production of methylenomycin A and actinorhodin by *Streptomyces coelicolor* has been elaborated in detail. For example, the genes for the methylenomycin are carried on the plasmid SCPI (*25*), whereas actinorhodin synthesis is determined by chromosomal genes (*25*). The biosynthesis of the aminoglycosidic antibiotics kanamycin, neomycin, and paromycin is assumed to involve the participation of a plasmid which determines the biosynthesis of deoxystreptamine, a common precursor of these antibiotics (*26*). Results obtained with istamycin indicate that plasmids may participate in catabolite regulation by glucose and in membrane permeability. Plasmids have been studied in other *Streptomyces* strains that produce antibiotics of the neomycin series, particularly in connection with the "modifying" enzymes—glycoside-3′-phosphotransferase and aminoglycoside-acetyltransferase, whose activities cause the inactivation of these substances (*27*). The presence of plasmids in strains that produce the macrolide antibiotics has shown that plasmid genes are involved in the expression of antibiotic resistance and in melanin formation (*22*).

A strain of *Streptomyces kanamyceticus* for industrial use, when propagated on a complex liquid medium and then plated on agar medium, was found to generate a low frequency (0.2 to 1 percent) of small raised colonies having a soft fragmented texture which have lost their capacity to synthesize kanamycin (Km^-) (*28*). Growing sonicated mycelia in the presence of acridine orange (AO), ethidium bromide (EB), or acriflavin (Acr) resulted in a higher frequency (10 to 20 percent) of Km^- colonies. Incubation of Km^- colonies at high temperature (35°C) in the presence of Km^+ colonies also resulted in a high incidence (90 percent) of the Km^- phenotype. The Km^- phenotype also developed when conidia from streak cultures were grown on oatmeal agar containing AO, Acr, or EB, where frequencies of 70 to 80 percent Km^- colonies were routinely obtained. The Km^+ colonies were found to be more resistant to kanamycin and amikacin (BB-K8) and than Km^- colonies, and the addition of kanamycin to oatmeal agar resulted in complete suppression of conidia of the Km^- type. The Km^+ isolate also produced higher amy-

lase activity in fermentation broths than the Km^- isolates. Selected Km^- isolates produced kanamycin when grown in media supplemented with deoxystreptamine, streptamine, or D-glucosamine, but not with streptidine: 2,6-dideoxystreptamine, 2,5,6-trideoxystreptamine, or 6-aminoglucose. Similar results were obtained with other plasmid-cured strains of *S. kanamyceticus* (*24*). These data are consistent with the hypothesis that active kanamycin production and other characteristics may be controlled by a plasmid gene (or genes) in *S. kanamyceticus* (*29*), and that inclusion of kanamycin in growth media appears to maintain the integrity of cells containing plasmids possibly by suppressing the growth of Km^- cells. Incorporation of kanamycin in the vegetative inoculum stage also appears to stimulate kanamycin production in laboratory fermentations.

The development of a clearer understanding of the role of plasmid genes in antibiotic production will open possible strategies for improving production in such strains. Manipulation of plasmid copy number, and plasmid gene regulatory elements, such as specific gene promoter DNA sequences, result in positive future results.

Recombination and Recombinant DNA Techniques for Novel Antibiotics

In addition to the manipulation of microorganisms by mutation-rational selection methods to improve antibiotic productivity, genetic recombination techniques can be applied in a directed manner to create desirable genetic alterations in microorganisms that may then synthesize novel hybrid antibiotics. In a general sense, genetic recombination methods include those techniques that produce organisms having stable, expressable, genetic traits obtained by combining genetic elements from two or more parent organisms. This definition includes transformation methods, phage-mediated transduction, plasmid-mediated conjugation, protoplast fusion, parasexual breeding (in fungi), and application of the recombinant DNA method, which overlaps other methods to some extent in that it involves transformation of cells with laboratory engineered specific recombinant DNA molecules via plasmid or phage vectors. It is possible with these (and other) methods to create new antibiotic-producing organisms having permanently altered genomes, with the hope and intent of either improving antibiotic production or generating new, hybrid antibiotic biosynthetic capabilities.

Among the potentially more promising recombinational methods for developing new antibiotics are (i) protoplast fusion to select for intraspecific recombinant strains that produce new antibiotics, (ii) self and shotgun gene cloning to generate new antibiotic synthetic pathways on a semirandom basis, and (iii) directed gene cloning to provide an added step or steps to an antibiotic synthetic pathway in a specific and highly controlled manner.

Protoplast fusion for new antibiotics. Species of *Acremonium chrysogenum* that overproduce cephalosporin C were obtained (*19*) by protoplast fusion methods for the development of genetic (haploid) recombinants by intraspecific fusion. The use of protoplast fusion for developing hybrid drugs is enhanced by the possibility of interspecific genetic recombination. Interspecific hybrid formation via protoplast fusion was achieved between species of *Aspergillus* and *Penicillium* (*30*). Fusions between distantly related species have been achieved, an indication that genetic recombination is possible given that morphological and metabolic properties of the hybrids are derived from both parent strains (*17*). Protoplast fusion hybrids of *P. chrysogenum* and *A. chrysogenum* were obtained, and they showed in some cases *Acremonium* morphology with *Penicillium* outgrowth, suggesting nutritional complementation. Stable isolates were obtained that had *Acremonium* morphology but differed in their response to phenoxyacetic acid as compared to the *Acremonium* parent in shake-flask fermentations. The prototrophy and altered morphology of these stable fusion products suggests that gene recombination between *Acremonium* and *Penicillium* has occurred in the hybrids (*17*).

Protoplasts of two *Streptomyces* strains, *S. griseus* which produces streptomycin, and *S. tenjimariensis* which produces istamycin, were fused and allowed to revert to a natural filamentous state on a selection medium (*31*). A fused hybrid was selected that produced a new antibiotic differing from the metabolites produced by either of the parents.

The development of recombinant DNA technology and techniques for transformation and transfection of protoplasts as well as intact organisms has made possible the exploitation of these methods for gene cloning in actinomycetes and fungi. The rationale for attempting shotgun and self-cloning is that transformants may acquire new genes for enzymatic activities that can modify the chemical structure of secondary metabolites normally produced by the organism to generate new antibiotics.

The basic requirements for utilization of the recombinant DNA method to transfer and express segments of foreign

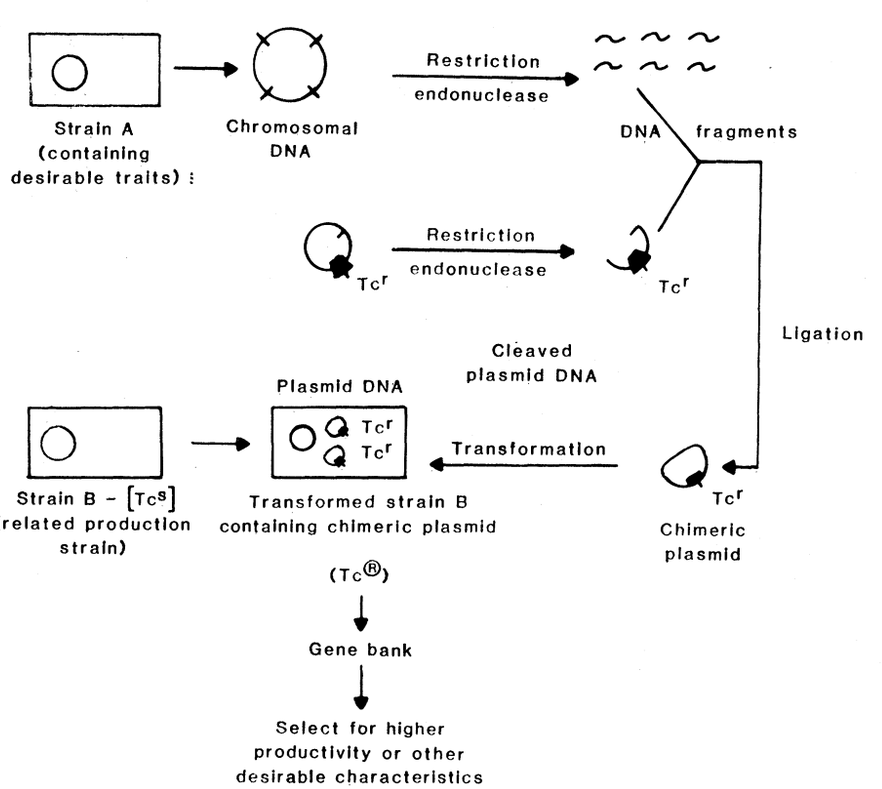

Fig. 4. Schematic diagram illustrating the self-cloning technique.

DNA in a host organism include (i) an appropriate vector DNA molecule (plasmid or phage) compatible with the cell and carrying appropriately localized control elements such as promoters and ribosome binding sequences; (ii) a convenient method for preparing the foreign DNA, cleaving it to a reasonable size range, and splicing into the vector DNA molecule such that it will have a good probability of being expressed should it be a gene; (iii) a method for introducing the recombinant DNA molecules into the host cell so that it is transformed to a new phenotype that includes the properties coded for by the recombinant; and (iv) a method for assaying for the expression of the desired gene products.

Shotgun cloning as used here refers to the transformation of a host organism with a gene library prepared with randomly fragmented DNA from a different donor organism. The library is carried in a vector compatible with the host organism and includes, in a random array, the entire genome of the donor organism. Hopwood (*5*) discusses aspects of genomic library construction with respect to streptomycetes. He shows that the number of clones needed in a library in order for an entire genome to be represented is a function of both the length of the cloned fragment and the genome size. It is best to use large-sized DNA fragments to construct libraries within the size limits imposed by the ability of the vector to form recombinants. Self-cloning is an example of shotgun cloning where the host and donor species are closely related (Fig. 4).

A major concern in the use of shotgun and self-cloning to generate new hybrid antibiotics is related to the magnitude of the screening effort. If a library from a donor organism is of size L (say 10^3 clones) then L (10^3) colonies will have to be screened when the library is used to transform one host. If cloning combinations are made, then all combinations of n strains cloned pairwise into one another would give $n(n\text{-}1)L$ clones to be examined for novel antibiotic activities (*5*). This number can grow rapidly, and hence considerable creativity and effort must be brought to the development of screening methods.

Shotgun and self-cloning techniques have been used to clone antibiotic resistance genes in streptomycetes (*9*). These experiments demonstrate the potential to mobilize and obtain expression of genes within and between species toward the rapid intermixing of genomes of antibiotic-producing microorganisms, thus increasing the probability and efficiency of new antibiotic discovery.

The use of the recombinant DNA method to isolate specific genes coding for proteins having particular activities has been well established in several biological systems. The potential applications of this method to solve specific problems related to antibiotic production are numerous. Genes coding for enzymes could be cloned into antibiotic-producing organisms to attempt to add one or two steps to existing biosynthetic pathways. Such alterations, although usually minor in terms of the overall physiology of the organism, could be of major economic value leading to large cost reductions of antibiotic production.

The rapidly developing recombinant DNA technology now provides approaches which may result in the successful transfer of acyltransferase genes from *Penicillium* to *Acremonium*, thereby resulting in a strain capable of synthesizing solvent extractable cephalosporins (*32*). The transfer of hydroxyaminobutyric acid acylase genes from butirosin-producing strains of *Bacillus circulans* to *S. kanamyceticus* could lead to the direct, efficient synthesis of the important semisynthetic aminoglycoside antibiotic, amikacin.

Recombinant DNA technology is also undergoing rapid development in streptomycetes, which produce 60 percent of the known antibiotics (*33*), therefore, the development of DNA cloning and self-cloning systems (Fig. 4) for *Streptomyces* would facilitate the genetic analysis for specific antibiotic biosynthetic pathways and of molecular mechanisms involved in their differentiation. An effective DNA cloning system for interspecies gene transfer in a number of differing antibiotic-producing species of *Streptomyces* has been reported (*34*). Thompson *et al.* (*35*) have described newly constructed cloning vectors from streptomycete plasmid and genomic DNA termed pIJ 101 and pIJ 61 which provide for both replication and transfer functions. pIJ 101 is a multicopy (40 to 300 copies per cell) plasmid with a broad host range occurring in *S. lividans* 5434. Moreover, the plasmid is self-transmissable and can mobilize chromosomal genes (*36*). Katz *et al.* (*37*) have used these plasmids to transform protoplasts of *S. lividans* 1326 and obtained expression of the gene coding for the synthesis of the enzyme tyrosinase.

Conclusions

There are many options available for altering the genetics of antibiotic-producing microorganisms to realize particular goals. Rational selection methods based on an enhanced understanding of the biosynthetic pathways for antibiotic synthesis can lead to radical improvements in strain productivity. Protoplast fusion technology can be used to allow genetic recombination to occur among different species of antibiotic producers to generate mixed synthetic pathways that may in turn give rise to hybrid antibiotics. Recombinant DNA methods can be used to clone genes for strain improvement and novel antibiotic synthesis into existing microorganisms in either a semi-random or specific manner. The coming decade promises to be filled with excitement and rewards as many of the ideas discussed here and elsewhere are implemented in the laboratories, pilot plants, and ultimately, production systems of the pharmaceutical industry.

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New Applications of Microbial Products

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Summary. Microbial secondary metabolites are now being used for applications other than as antibacterial, antifungal, and antitumor agents. These applications include use against parasites (coccidia, helminths) and insects as well as for animal and plant growth stimulation, immunosuppression, uterocontraction, and other pharmacological activities. Further applications are possible in various areas of pharmacology and agriculture, a development catalyzed by the use of simple enzyme assays for screening prior to testing in intact animals or in the field.

The expression "wonder drugs," refers to the selective action that microbial chemicals exert against pathogenic bacteria, fungi, and tumors. The discovery of this selective activity ushered in the "antibiotic era," and for more than 40 years we have been the beneficiaries of this remarkable property of antibiotics. The success rate has been so high that for years the predominant application of microbial secondary metabolites was that of antibacterial, antifungal, and antitumor chemotherapy. Unfortunately, however, such a restricted view of the potential of microbial idiolites (secondary metabolites) has retarded the further development of the fermentation industry. Many industrial microbiologists have felt that antibiotic activity is merely the tip of the iceberg; that is, with regard to the potential application of microbial secondary metabolites for the benefit of humankind, the surface has only been scratched. In this article, I point out those cases in which microbial metabolites have surprising applications and also point to some challenges for the future. In this way, I hope to engender further appreciation of Jackson Foster's astute and predictive statement: "Never underestimate the power of the microbe" (*1*).

Antiparasitic Activities

One of the major economic diseases of poultry is coccidiosis, which is caused by species of the parasitic protozoan *Eimeria*. For years, this disease was treated solely by synthetic chemicals, and indeed only synthetic compounds were screened for coccidiostat activity. Although they were generally effective, resistance developed rapidly in the coccidia, and new chemical modifications of the existing coccidiostats had to be made. Then a parenterally toxic and narrow-spectrum antibiotic, monensin, was found by the group at Eli Lilly & Company to have extreme potency against coccidia (*2*). At first there were grave doubts that the fermentation process for this polyether compound could be improved to the point where monensin would become economically feasible. However, industrial genetics and biochemical engineering techniques were applied to this improvement project, and as a result the polyethers (*2a*), especially monensin (produced by *Streptomyces cinnamonensis*) and lasalocid (produced by *Streptomyces lasaliensis*), now dominate the commercial coccidiostat market.

An interesting sidelight of the monensin story is the discovery of its further use as a growth promoter in ruminants. For years, synthetic chemicals had been screened in an effort to supplement cattle and sheep diets with an agent that would eliminate the wasteful methane production and increase volatile fatty acid formation (especially propionate) in the rumen, thus improving feed efficiency. Although the concept was sound, no useful products resulted. Experimentation with monensin showed that polyethers have this beneficial activity, and now these compounds are widely used (*3*). Polyethers also have cardiovascular effects that are being studied for possible medical application.

Another major agricultural problem has been the infection of farm animals by worms. The predominant screening effort over the years was the testing of synthetic compounds against nematodes, and commercial products did result. Certain antibiotics had also been shown to possess antihelminthic activity (for example, hygromycin, antibiotic G-418, destomycin, paromomycin, antibiotic complex S15-1, antihelvencin, aspiculamycin, anthelmycin, myxin, thaimycin, and axenomycin) against nematodes or cestodes (*4*), but these failed to compete with the synthetic compounds.

Although the Merck Sharp & Dohme Laboratories had developed a commercially useful synthetic product, thiobenzole, they had enough foresight to also examine microbial broths for antihelmin-

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