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CHAPTER 8

Two Decades of Strain Development in Antibiotic-Producing Microorganisms

R. P. ELANDER

Antibiotic Manufacturing and Development Division
Eli Lilly and Company, Indianapolis, Indiana

Mutation and selection is one of the most successful methods employed for improvement of antibiotic yield. Indeed, the greatest single factor contributing to large-scale penicillin manufacture was the development of potent mold strains. In this, strain development was firmly established. The mutation-selection approach for improved yield is still predominantly employed in industrial laboratories today.

During the past two decades, microbial genetics has grown from obscurity to one of the most exciting areas of biology. As a consequence, many new philosophies and approaches are now available to industrial strain development programs. These include the application of potent new mutagens, and, for many of them, a molecular basis for their action; the use of antimetabolites and the directed isolation of superior yielding variants; the incorporation of specfic analogues by mutant strains and subsequent antibiotic modification; the production of structurally-modified antibiotics by mutants with specific gene blocks; the selection of modified streptomycete strains following phage attack; and the discovery of sexual as well as parasexual mechanisms in actinomycetes and "imperfect" fungi. There is now speculation concerning interspecific recombinants and the development of strains with increased ploidy for use in qualitative and quantitative antibiotic change.

These developments stress the continued importance of strain selection in the industrial environment. Much work is necessary to make the more sophisticated techniques flexible in the industrial laboratory. The industrial microbiologist will have to be well trained in the concepts and practice of microbial genetics. During the next decade, it would appear that applications of microbial genetics will play a more and more important role in industrial strain development.

INTRODUCTION

This report forms, in reality, the background for the improvement of every microbiological process. Improvement of productivity becomes necessary after the initial selection, evaluation, and discovery of a useful microbial product. At the research stage, when a potentially promising drug is under evaluation in the laboratory and clinic, a dependable supply of the drug is necessary. During a recent evaluation of a promising antibiotic for tuberculosis, a supply of 250 kilograms of pure drug was required for clinical trials alone. Contrast this with the early clinical work on penicillin in England by the Oxford group. According to Florey et al. (1949), the total world supply of penicillin (purity approximately 3%) available for clinical trials for the year 1942 was less than 122 million units or approximately 73 grams as pure penicillin G. This represented the total penicillin production by a number of American and British firms. The scarcity of material for trial studies is emphasized by the fact that initially the patient's urine was collected and the drug re-extracted and used again. Such a procedure also yielded a material of considerably greater purity.

Detailed programs concerned with strain selection, maintenance, and improvement

usually commence shortly after favorable reports are received from clinicians suggesting clinical efficacy. The history of penicillin illustrates the importance of effective strain improvement. Today, after two decades of research, microbiologists are still actively concerned with the improvement of strains of *Penicillium*. Thus, the material for this paper and this symposium is current and, indeed, one of the most fascinating and rewarding areas of antibiotic research.

Recently, Alikhanian (1962) reviewed the advances made in Russia on several important antibiotics through strain selection techniques. He cited penicillin increases from 20 units/ml in 1943 to 8,000 units/ml in 1955. For erythromycin, he reported increases from 100 units/ml in 1955 to 2,000 units/ml in 1961. Similar, if not more impressive, advances could be recorded for non-Russian technology. However, competitive pressures restrict publication of such information.

The growth of the antibiotics industry in the United States over the past two decades can be reviewed. According to the U. S. Tariff Commission Report for 1945, the total United States production of penicillin was nearly 7.5 trillion units (4.5 metric tons). The total sales for the same period was 46.5 million dollars ($11/gram). It should be remembered that at this time, penicillin was the only commercial available antibiotic. In contrast, the latest available compilation revealed the total units of penicillins manufactured in the United States for 1963 was 715 trillion units (480 metric tons) representing 72 million dollars (15 cents/gram) in sales at wholesale prices. For the same year, the total world production of all antibiotics reached a peak of 6.7 million pounds (3050 metric tons) valued at 388 million dollars. One of the major factors contributing to the tremendous gains in antibiotic production and economics, over these past two decades, is the field of research devoted to increased productivity through strain development.

There are many methods for effective strain improvement. Initially, strain selection was dependent upon the degree of spontaneous variability encountered in natural spore populations of mold and streptomycete organisms. The concept of variation had been firmly established in microbiology long before the advent of the antibiotic era. However, by the late forties, a number of effective mutagenic agents were known, and certain of these were finding their way into strain development laboratories. Although mutation was generally considered to indicate loss of function, it was challenged repeatedly by the discovery of enhanced variants (gain mutants) isolated from conidial populations exposed to radiation and chemical mutagens. However, there is no evidence that increased production is not a result of decreased function of some enzyme system. As a consequence, the mutation-selection process is now undoubtedly the most important method for obtaining improved strains.

With the rapid advances in the field of genetics and molecular biology during the fifties and sixties, many new and sophisticated techniques became available to specialists in strain development. The discovery of diverse recombination mechanisms in streptomycetes and the more recent application of parasexual concepts to industrially important "imperfect" fungi are illustrative of this potential for new strain development technology. Unfortunately, no clear-cut application of these ideas is available in the published literature. Most of the recent research in microbial genetics is confined to organisms of low economic interest. Although generalities frequently appear in print, peculiarities of organism and strain make their application difficult. Another important aspect concerning the application of the new technology depends on the strain selection specialist becoming familiar with and then applying these new techniques to the problems of his industrial environs.

An important purpose of this review, and, indeed, this symposium, is to describe the

philosophy and techniques of strain development as they have evolved over the past two decades with certain comments regarding their practical application. A projection of the future for strain development during the next two decades will also be presented.

The Selection of Strains as Spontaneous Variants

Fortunately, or unfortunately, antibiotic-producing microorganisms exhibit a great capacity for natural variation. Therefore, the selection of strains from natural spore populations is of great practical importance in strain development. Undoubtedly, its greatest application is in the maintenance of improved antibiotic-producing cultures. Heterokaryosis and subsequent culture rundown (Haas et al., 1956), despite the introduction of vastly improved preservation methods, is still an important problem in commercial culture laboratories. The continued selection for preferred colony type is a never-ending process and one of the major factors responsible for maintenance of production at constant high levels.

Natural selection for improved strains is also important in the early stages of development programs. Most mycelial organisms are probably heterokaryotic when freshly isolated from their natural environment. Selection of single spores from heterokaryotic organisms with uninucleate conidia often leads to discovery of diverse homokaryotic colony types, many of which may represent superior antibiotic-producing entities. In the early stages of development, one discretely selects highly-conidiating, stable (non-sectoring) isolates and proceeds to evaluate them for desirable characteristics; notably, antibiotic species or titres. An example of direct selection was the isolation of *Penicillium* variants which synthesized copious amounts of penicillin G in submerged fermentation (Raper, 1946).

While advances in productivity can be obtained by direct selection techniques, the use of strains subjected to mutagenic physical and chemical agents greatly increases the probability of discovery of improved strains (*see* Brown and Elander, Ch. 14). Such techniques were also responsible for the selection of non-pigmented variants of *Penicillium* (Backus and Stauffer, 1955) and, indeed, for mutants capable of synthesizing antibiotics with structural modification (Ballio et al., 1960).

The Selection of Strains as Induced Variants

Thom and Steinberg (1939) were probably the first to apply mutation methods for the improvement of mold strains. In the middle forties, intensive development programs on penicillin led to the adoption of mutation and selection as a major tool in strain improvement. The pioneer work of Backus and Stauffer (1955), at the University of Wisconsin, provided the major source of high-yielding *Penicillium* cultures. Today, most, if not all, strains employed for the manufacture of penicillins have their origin in one of the members of the "Wisconsin Family." Mutation methodology as applied to antibiotic-producing organisms are described in articles by Darken et al. (1960) on tetracycline; Backus and Stauffer (1955) on penicillin; Dulaney, Ruger, and Hlavac (1949) on streptomycin; and Elander, Stauffer, and Backus (1960) on cephalosporin. Reviews by Nelson (1961), Alikhanian (1962), and Calam (1964) offer valuable information on this subject.

The Role of Major Mutation in Strain Development

The concept and role of mutation as applied to industrially-important microorganisms has two aspects. The first, major mutation, involves the selection of mutants which

manifest a pronounced change in a biochemical character of practical interest. Such variants are commonly used in genetic studies and may be classified, appropriately or inappropriately, as loss mutants. Such variants are isolated routinely from populations surviving prolonged exposure of a mutagen. In contrast, minor mutants show only subtle change in a particular character. In fact, the changes are so slight that usually the variants are not morphologically distinguishable from parent strains. Such mutants are common in all of our important antibiotic-producing organisms.

Examples of major mutation in strain development are numerous. The case of the important high-yielding, non-pigmented penicillia has already been mentioned. Alikhanian (1962) cites a role for major mutation in the streptomycin-producing organism, *Streptomyces griseus*. The initial strain synthesized in large amounts a substance with low activity in addition to small quantities of streptomycin. The substance is mannisidostreptomycin (streptomycin B), which competes with streptomycin for biosynthetic intermediates and also interferes with efficient isolation of the antibiotic. A variant was finally isolated which produced negible amounts of the undesired substance, thus allowing for greater synthesis and recovery of the desired moiety.

A careful study of variants exhibiting impaired antibiotic productivity may elucidate biosynthetic pathways and contribute to the identification of precursors. Studies by Miller et al. (1965) on tetracycline and Barchielli et al. (1960) on cobalamin reveal interesting data on precursor molecules involved in reaction steps prior to terminal ring closure. Heterokaryons also offer unique systems for the study of biosynthetic pathways. Heterokarotic strains capable of elaborating antibiotics may be synthesized from auxotrophic strains with impaired antibiotic activity. Study of the impaired homokaryotic strains may reveal precursor accumulation.

Ballio et al. (1960) using variants of *Penicillium chrysogenum* (Wis. 51-20) studied changes in the antimicrobial spectrum penicillins following incorporation of certain α-ϵ-dicarboxylic acids. Upon the addition of adipic acid, one variant was shown to elaborate a new penicillin (4-carboxy-n-butyl penicillin). This antibiotic is very similar to penicillin N (cephalosporin N), both in structure and antimicrobial activity.

In recent years, the use of major mutation has acquired particular significance and, in certain instances, has led to new and more efficacious products. The tetracycline-producing organisms appear to be particularly amenable to this approach. McCormick et al. (1957) described an interesting modification of tetracycline synthesized by a mutant strain of *Streptomyces aureofaciens*. The antibiotic was shown to be changed at the C-5a position and was almost devoid of antibiotic activity. They also reported that S. *aureofaciens* (strain S-604) synthesized 6-demethyltetracycline, a new antibiotic material not elaborated by the Duggar strain A-377. The new molecule has several advantages over the methylated form and, today, is one of the leading commercial forms of tetracycline.

The employment of mutants for the synthesis of modified antibiotic molecules appears to be a fertile area for major mutation in strain development. Mutants have been mentioned already that may accumulate precursor molecules which aid in the elucidation of pathways. An insignificant reconstruction of an antibiotic molecule could lead to new biological and therapeutic properties of a known antibiotic. Some years ago, Kelner (1949) published a paper which has great interest in this connection. He examined a series of streptomycete cultures which failed to inhibit certain test bacteria. The negative strains were then exposed to heavy doses of UV and X-ray radiation. The dose for the X-ray radiation was 300,000 r and non-irradiated strains served as controls. After examining several thousand irradiated strains, Kelner found mutant lines

which exhibited antimicrobial activity. In fact, certain weak antibiotic producers then showed a significant change in spectrum. The difference in antimicrobial spectrum indicated that in certain cases this change might result from qualitative modification of an antibiotic. This has been well documented for terramycin and aureomycin. Thus, major mutation may lead to numerous new structurally modified antibiotics. Such techniques may also be important for the screening of microorganisms which elaborate non-detectable quantities of an important antimicrobial. Mutants may elaborate detectable amounts of the substance. This tool could serve as a valuable aid in screening for new antibiotics.

The Role of Minor Mutation in Selection

Minor mutation usually plays the dominant role in strain development. By definition, these mutations affect only quantitatively the amount of product of interest synthesized. Such mutations are subtle, and variants exhibiting such features are usually similar phenotypically to the parent form. They show rapid and abundant mycelial and conidial development and produce only slightly more antibiotic than the preceding parental strain. Quantitative definition of a significant yield increase is somewhat relative and dependent on productivity of the parent. Usually, a 10 to 15% increase is implied. The variants are usually selected from conidial populations exposed to small or moderate doses of a mutagen. If one repeatedly isolates "minor" (positive) variants and uses each succeeding strain for further mutation and selection, after several stages, a significant yield increase may be obtained. Such increases have also been obtained without the introduction of mutagens. Thus, the problem of strain development with respect to improvement is, in reality, the problem of increasing the concentration(s) of hereditary factor(s) responsible for productivity in the original genotype. Were it not for the improvement or productivity through minor mutation, the cephalosporins, especially cephalosporin C, its nucleus (7-ACA) and derivatives, would still be laboratory curiosities (Florey, 1955).

Minor variants exhibiting slight change in a quantitative feature may vary also in other features owing to pleiotropic effects. Since such variation is slight, there is great dependence on efficient and accurate selection techniques. The population to be tested must be large and the assay must be accurate and specific for the desired product. Problems of this sort are primarily statistical and are discussed in articles by Davies (1964) and Brown and Elander (*see* Ch. 14).

Examples of gradual step-wise improvement in antibiotic production are numerous and may be seen in studies of penicillin type (Backus and Stauffer, 1955) and cephalosporin (Elander et al., 1960). The published data of Alikhanian (1962) on penicillin demonstrate well this philosophy of step-wise selection which has been followed in the United States for at least two decades. The continued selection of strains with only 10 to 12% greater penicillin productivity than the previous parent resulted in the selection of a variant which exceeded its original parent by 64%. Similar examples are commonplace throughout the antibiotic industry.

Many refinements in the techniques of mutation and selection have been introduced during the past two decades. Today, there are scores of mutagens available; and for many of them a mode of action is known. A recent review on the chemical basis of mutation is available (Orgel, 1963). Unfortunately, the phenomenon of mutation is random and not directable, so that mutation as applied to strain development is largely empirical. Little, if anything, is known of the kind of mutants desired. The limited

knowledge of antibiotic biosynthesis is shown by the fact that a biosynthetic pathway is not elucidated fully for a single antibiotic. There is a paucity of solid information available about the mechanism of biosynthesis for the first important antibiotic, penicillin.

In contrast, considerable refinement in mutation and selection techniques has been demonstrated over the past two decades. Refinements in evaluation have been numerous, and these probably represent the key to a successful strain development program. The importance of evaluation is illustrated by the problem of selecting relatively few enhanced variants from a population of hundreds or thousands of individuals showing control yields or less. Many of these techniques are peculiar to organisms of commercial interest, and their publication is restricted.

Examples of sophistication in techniques are numerous. One is the work of James, Rubbo, and Gardener (1956) who published an interesting method of selection with the citric acid-producing fungus, *Aspergillus niger*. The preliminary test involved the propagation of mold colonies on filter paper impregnated with a suitable production medium containing an appropriate indicator dye. After a prescribed incubation period, the acidity was measured as citric acid. The best strains were then evaluated in shake flask fermentations. Many of the strains which were superior on the preliminary test synthesized greater quantities of citric acid in submerged fermentation. Alikhanian (1962) studied colony morphology as related to subsequent productivity in penicillin fermentation and he recommended the rejection of poor growing, poor conidiating, strains of *Penicillium*. He also suggested screening variants of *Penicillium* in the absence of phenylacetic acid on the basis that the enzymes generating penicillin nucleus (6-amino penicillanic acid) might become rate limiting with respect to total penicillin G yield. He finally suggested the screening of new strains on an inferior medium, because a strain superior on such media might exhibit marked superiority under more optimal fermentation conditions. Ostroukhov and Kunznetsov (1963) described a unique plate method for the detection of superior penicillin producers on the basis of increased oxidation-reduction potential. They also mentioned that strains may exhibit more negative potential under conditions of reduced aeration.

A method based on a different rationale was discovered by Adelberg (1958) and later applied by Scherr and Rafelson (1962). An excellent recent application of this principle to amino acid fermentations is described by Karlström (1965). Adelberg reported that bacterial mutants resistant to amino acid analogues excreted small quantities of the homologous amino acid. This causes incomplete repression of enzymes synthesizing the amino acid. If it could be assumed that similar repression systems control antibiotic synthesis, and if antibiotics were essential metabolites for the organisms synthesizing them, mutants could be selected lacking repressors and thereby select superior antibiotic-producing variants. Moreover, antibiotics are generally large complex molecules containing unusual sugar moieties, etc., which are synthesized by complex and unknown pathways. Such antimetabolites could aid in the eluciation of biosynthesis, but the design for such molecules is still a subject for the future. The technique described above has been invaluable in the selection of superior vitamin and amino acid-producing variants. It should be emphasized that, the more accurate and sophisticated the screen, the better the probability of its success. A screen can only be as good as the design and the questions built into it. Sophistication at the selection stage often results in an effective strain development program. In summarizing the mutation concept as applied to strain development, certain generalities appear appropriate.

1) Strains selected as obvious variants following exposure to mutagens usually are in-

ferior in their capacity to elaborate antibiotics. Those strains with enhanced capacity for accumulation of antibiotics are extremely few in number, and selection and evaluation play extremely important roles in their detection.

2) Mutagen dose is important in strain selection methodology. The rate of mutation is a function of dose; hence, mutants sought for major mutation roles are best isolated from populations surviving prolonged doses of mutagens. Variants employed for increased productivity are generally isolated from populations surviving intermediate dose levels.
3) Strains with enhanced capacity for antibiotic synthesis generally exhibit wild-type morphology and growth habit. Strains with altered morphology, etc., may be inherently better producers but may require considerable fermentation development. Since positive variants are extremely few in number, it is better to screen a large number of variants on a few fermentation conditions rather than to screen a small number of variants under a wide variety of environmental conditions. This is especially true when one seeks only quantitative change in the variants.
4) The philosophy of step-wise selection implies small increments in increased antibiotic productivity. The range of increased productivity is generally 10 to 15% after the initial strains are obtained by natural selection. As productivity increases, the probability of finding superior strains decreases; hence, accurate and more sophisticated evaluation procedures play increasingly important roles. The development program is only as effective as the mutation, selection, and evaluation procedures coupled to it.
5) Variant strains often require special propagation and preservation procedures. Actual production gains depend largely in stability and reliability of performance. Maintenance through continued selection and purification plays an important role in production laboratories.
6) Although a strain may meet the numerous necessary criteria of superiority in the laboratory, there is no guarantee that enhanced productivity will occur in production fermentors. Aeration-agitation patterns, nutrient availability, etc., are often unbalanced for variant cultures. Scale-up experimentation through long-term pilot plant studies is often necessary before any enhanced strain potential may be realized in actual production.

Genetics and Strain Development

To date, the study of microbial genetics has been largely academic. Russian scientists have applied recombination methods to industrial organisms with some success during recent years. However, industrial microbiologists of the western world have either not been as successful or have not been free to publish their results. Although microbial genetics has grown to a high degree of maturity, it is still, by necessity, largely theoretical. The *Neurospora, Aspergillus,* and *Escherichia* systems still dominate the literature; and it will be some time before organisms of more economic interest are employed for genetic studies. Studies have been made on industrially important fungi, but their application to industrial problems is still remote.

The discovery of parasexuality in asexual molds by Pontecorvo and Roper in 1952 opened the door for planned breeding with economically-important fungi. The methodology as described in their patent (Pontecorvo and Roper, 1958) was intended to make possible the synthesis of new strains with increased capacity to synthesize economically-important fermentation products. The technique essentially consists of combining two

strains with certain properties to form a hybrid with more desirable properties. Complementary biochemical markers are sought in the strains and they are then propagated together on a medium lacking the required growth factors. This technique involves formation of a heterokaryon which contains nuclei of the two parents in a common cytoplasm. Such conditions favor formation and multiplication of heterozygous diploid nuclei in which the factors of both parental strains are combined. The cells, usually conidia, are easily recognized by color and/or biochemical markers. Although a number of recombinant strains are unstable, an occasional non-sectoring recombinant is found. Certain of these possess genomes differing both quantitatively and qualitatively from either of the original parent strains.

The first studies on genetic recombination in actinomycetes were reported with strains of *Streptomyces coelicolor* by Sermonti and Spada-Sermonti (1958). More detailed studies by Hopwood and Sermonti (1962) with the *S. coelicolor* system showed the existence of two linkage groups. They also reported that conidia with heterozygous nuclei are produced which, in turn, persist through several generations. Certain of the heterozygous nuclei (hemizygous) appear to be incomplete because of losses of chromosomal material after zygote formation. A similar situation exists in *Escherichia coli*, and this, again, suggests the close relationship of the streptomycetes to the eubacteria. One of the major differences between the stereptomycete system and the parasexual system in fungi is that stable heterozygous diploid nuclei are only rarely produced in streptomycetes. The diploid condition is especially stable in *Aspergillus (Eurotium) nidulans*. An excellent review of streptomycete genetics is now available (Hopwood and Sermonti, 1962).

Transfer of genetic material from host to donor cell via actinophage is well established. Phage-resistant isolates can be recovered which are often drastically altered in morphology and biochemical make-up. Alikhanian and Iljina (1958) reported on the mutagenic effect of phage on *Actinomyces olivaceus*. They claimed up to 99% of the resistant isolates represented drastically mutated forms. Recently, Alikhanian and Teteryatnich (1962) described the discovery of "trans-active" variants in *Actinomyces streptomycini*. They showed the ability to form streptomycin was transduced via phage to non-producing variants and that certain of these synthesized greater quantities of antibiotic than the original parent strains.

Genetic information may also be transferred to recipient cells by isolated desoxyribonucleic acid. While this phenomenon has been extensively investigated in bacteria, there are only three successful reports in actinomycetes and molds. Shamoian, Canzanelli, and Melrose (1961) reported "quasi-transformation" in *Neurospora* by crude nucleic acid preparations and Jarai (1961) demonstrated transformation in the tetracycline-producing organism, *Streptomyces aureofaciens*. Recently, Matselyukh (1964) reported studies on the transformation of antibiotic production to various actinomycetes by isolated DNA.

Genetic Studies with Specific Reference to Strain Improvement

The citric acid-producing organism, *Aspergillus niger*, was one of the first industrial organisms shown to possess a complete parasexual cycle. Pontecorvo, Roper, and Forbes (1953) described the formation of stable heterozygous diploid cells indicative of chromosome cross-over. Later, Japanese workers reported the existence of parasexual mechanisms in certain koji molds including *Aspergillus orzyae* and *A. sojae* (Ishitani, Ikeda, and Sakaguchi, 1956). Ikeda et al. (1957) described superior kojic

acid production by certain diploid strains and showed that interspecific recombination between the two species was possible. Triploid and tetraploid strains were also shown to be better producers of protease and amylase.

The most extensively studied parasexual system in antibiotic-producing molds is that of *Penicillium*. Pontecorvo, Roper, and Sermonti (1954) reported the existence of the complete cycle in this fungus. Later, Sermonti did extensive and more definitive work at Rome. His first papers described the formation of diploid and segregant types, while later publications dealt with chromosome mapping and various aspects of genetic control of penicillin production.

Sermonti described, in a series of papers, penicillin production by diploid segregants and auxotrophic diploid segregants. Such strains varied widely in antibiotic yield. Generally, although an occasional good producer was found, the strains were inferior to the parents in productivity. Diploid segregant strains fell into two distinct classes, usually resembling one or the other parent. This "parental genome-segregation" turned out to be a major obstacle to planned breeding in *P. chrysogenum*. Sermonti (1961), in summarizing his work, took a pessimistic view of the application of parasexuality to strain improvement. He regarded the long time build-up in productivity in *Penicillium* as the result of a complex polygenic balance of mutant alleles, rather than of an additive effect of favorable mutations.

During this period, the Russians were investigating the same genetic system in Moscow. Alikhanian (1962) described a "New Hybrid" capable of synthesizing over 4,000 to 6,000 units/ml. Continued hybridization resulted in recombinants capable of synthesizing even higher yields. Alikhanian differs in opinion with Sermonti and, perhaps more realistically, considers some aspects of the parasexual process as applicable to industrial fermentations. He believed that parasexual recombination made certain strains more amenable to mutation-selection programs and advised the introduction of parasexual techniques when conventional strain selection methods failed to give the desired enhanced variants. The author feels that a balanced program employing both methods is most successful. Since the introduction of biochemical mutation tends to reduce productivity, the use should be employed of mutants impaired at loci not affecting antibiotic synthesis. In other words, one should use biochemical mutants which yield good antibiotic titres before starting the parasexual work.

Recently, Macdonald (1964) and Macdonald, Hutchinson, and Gillett (1963, 1964) published a series of papers on the application of parasexuality to industrial processes. They intensively studied certain diploid strains giving 20% higher penicillin yields than the parent. However, their yields were low and ranged from 3,000 to 4,000 units/ml. More noteworthy was the discovery of four linkage groups and the suggestion that leucine-less segregants gave good productivity. They also found that pleiotropy was involved in cases of decrease or loss of penicillin productivity. Their results confirmed Sermonti in that penicillin production was under nuclear control with some possible cytoplasmic influence. Pigment production was also shown to be under nuclear control, and this capacity appeared to be a dominant trait. Parental genome segregation was again shown to be a barrier. Also, they suggested the use of mutagens for inducing segregation in certain desirable diploid strains.

One of the major problems encountered in *Penicillium* is the instability of the diploid strain. This weakness, perhaps more than any other single factor, prevents more application of parasexual concepts to penicillin methodology. Macdonald (1964) suggested inducing chromosome rearrangement in the cultures by using heavy doses of radiation such as X-ray. Such rearrangement may restrict recombination, thereby

minimizing segregation. Dwarf or restricted growth mutants may be employed for recombination. Diploid recombinants may be selected and when parental segregants are formed, they will be contra-selected in a complex production medium.

The author and associates have spent considerable time in adapting parasexual techniques to *Penicillium*. Complementary diauxotrophic strains isolated from a current production strain were mated on a limiting nutrient medium and heterozygous diploids were recovered along with their segregants. Most auxotrophs produced only 25% of control yield, but in certain diploid strains productivity was nearly restored to the level of the parent control. In fact, some diploids exceeded the parent strain in productivity in shake flask fermentation. An interesting consequence of this work was the discovery of an extremely stable diploid strain. Population studies showed less variation in colony type than in wild-type production strains. Mutation techniques were finally applied to induce segregation of this diploid. Diploid strains which are stable in culture tend to be the better producers in our laboratories.

Genetic approaches have also been applied to cephalosporin-producing fungi. Fantini (1962) did a thorough study on *Emericellopsis,* a homothallic ascomycetous fungus. Parasexual as well as sexual phenomena were investigated and correlated with cephalosporin N (a penicillin) production. Recombinant types and diploids were inferior in productivity to the wild-type parent stock. A notable outcome of Fantini's study was the isolation of a recombinant which possessed antifungal activity. Curiously, such activity was absent in the parental strains. He concluded from this study that genetic approaches may best apply to the problem of modifying the chemical nature of the metabolic products under investigation.

Recombination studies in a number of economically-important streptomycetes have been described. The studies include published reports on *S. fradiae, S. griseus, S. griseoflavus,* and *S. erythreus*. In 1961, Mindlin, Alikhanian, and Vladimirov described a breeding program with certain oxytetracycline-producing strains of *S. rimosus*. Originally, their program was designed to select a recombinant with decreased foaming characteristics on a high carbohydrate, high protein-containing medium. The strain finally selected, L-S-T-Hybrid, not only possessed this characteristic but also yielded a significant increase in yield of oxytetracycline. Considerable research has also been undertaken with *Streptomyces aureofaciens* (Alikhanian and Borisova, 1961). They obtained recombinants with increased productivity and certain of their argininine-less mutants lost the ability to synthesize chlorotetracycline. Huang-Lo (1962) discovered evidence for recombination in the macrolide-producing organism, *Streptomyces erythreus*. Certain recombinant strains synthesized excellent titres of erythromycin. Others exhibited a wide range of antibiotic yield.

The use of actinophage not only as a "mutagen" but also in transduction studies has had extensive application in certain commercial strains of streptomycetes. Phage attacks still plague antibiotic producers, and screening for phage resistant variants is a common practice in industrial laboratories. Resistant variants isolated from lysed tank material may synthesize more antibiotic than the sensitive forms. Alikhanian and Teteryatnik (1962) reported the formation of streptomycin-producing variants from a non-producing LS-1 strain of *A. streptomycini* through the action of actinophage. One transactive variant synthesized 5,200 units/ml of antibiotic compared to 4,100 units/ml for the parent strain. The author and associates have recently applied phage techniques to certain high-producing strains of the erythromycin organism, *Streptomyces erythreus*. In certain strains the phage system was more successful in obtaining mutants with desirable properties than conventional mutation-selection methods.

DNA base composition has been extensively studied in actinomycetes in recent years. Jones and Bradley (1963) and Frontali, Hill, and Sylvestri (1965) studied numerous *Streptomyces* and *Nocardia* strains and reported mole percent guanosine:cytosine ratios from 74.4 to 78.5 using several sophisticated methods. Their data suggested close taxonomic relationships within the genus. More importantly, the possibility of intragenic recombination was further strengthened by such data. The discovery of polyvalent phage also was suggestive of a close relationship.

Several laboratories have recently reported interspecific recombination in *Nocardia* and *Streptomyces*. Adams (1964) reported in *Nocardia* a system closely resembling classic heterothallism. A most important paper by Alacevic (1963) describes interspecific recombination between various antibiotic-producing entities. Recombinations between *S. rimosus* and *S. coelicolor, S. rimosus* and *S. aureofaciens* and between *S. aureofaciens* and *S. coelicolor* again suggest the close relatedness of the species. No statements were published concerning the elaboration of structurally-modified antibiotics. One need scarcely belabor the importance of this concept to the antibiotic industry. Bradley (1964) reported interspecific recombination between *S. aureofaciens* ATCC-10762 and *S. violaceoruber* S-199. The interspecific recombinants were phenotypically similar to *S. violaceoruber*. They were unstable in culture and produced very little antibiotic.

The Next Two Decades

During the past two decades, strain development has grown into an indispensible unit in the antibiotic industry. Indeed, in many respects, it is responsible for the extraordinary growth of the industry and has been the means of more efficient production and subsequent cost reduction needed to meet the huge market demands experienced throughout the world. Despite the size of the market and the ever-growing demand, competition is keen and, therefore, continued stress must be on increased productivity. On this basis, the future of strain development is secure. Indeed, it will be secure as long as antibiotics are employed for the treatment of disease.

The methodology and, indeed, the philosophy of strain development has undergone extensive changes and has matured since the early forties. The basis for these changes stems from the rapid advances made in microbial genetics and the new molecular biology. There is every reason to believe that this new biology will continue to advance at an even more rapid pace during the next two decades. In turn, new and undoubtedly more sophisticated techniques will be available to specialists in strain development. It will become an even greater necessity for the industrial microbiologist to keep abreast of his field. Each success employing genetic methods in strain development will depend largely on the ability of the strain development specialist to modify and tailor the methodology to the peculiarities of the organism of commercial interest.

Throughout the course of this review, many predictions were made about the future of strain development during the next two decades. The potential of the new strains produced through the techniques of transformation, transduction, and the vast array of recombination methods will offer numerous interesting avenues of application and may culminate in products now not known. The development of higher ploidy strains in antibiotic-producing fungi may lead to unparalleled productivity of current products, new antibiotics, and other microbial products. Perhaps the greatest potential for the future lies in the area of molecular modification through genetic approaches. Look only at the penicillins to see the opportunities in this field of endeavor. Interspecific

and possible intergeneric recombination also offer exciting new antibiotic research. The use of polyvalent phage and transduction techniques along with recombination methods may lead to the discovery of new antibiotics effective against resistant bacterial pathogens, fungi, and viruses responsible for plant, animal, and human disease.

In summary, the next two decades may well be the golden era for strain development and applied microbial genetics.

LITERATURE CITED

Adams, J. N. 1964. Recombination between *Nocardia canicruria* and *Nocardia erythropolis*. *J. Bacteriol.* **88**:856-876.

Adelberg, E. A. 1958. Selection of bacterial mutants which excrete antagonists to antimetabolites. *J. Bacteriol.* **76**:326-328.

Alacevic, M. 1963. Interspecific recombination in *Streptomyces*. Nature **197**:1323.

Alikhanian, S. I. 1962. Induced mutagenesis in the selection of microorganisms. *Advan. Appl. Microbiol.* **4**:1-5. Academic Press, New York.

Alikhanian, S. I., and L. N. Borisova. 1961. Recombination in *Streptomyces aureofaciens*. *Sci. Rep. Ist Super. Sanita* **1**:470-472.

Alikhanian, S. I., and T. S. Iljina. 1958. Mutagenic action of actinophages. *Nature* **181**: 1476-1477.

Alikhanina, S. I., and A. F. Teteryatnik. 1962. Formation of streptomycin-producing variants from mutants of *Actinomyces streptomycini* strain LS-1 through the action of actinophages. *Mikrobiologiya* **31**:54-60.

Backus, M. P., and J. F. Stauffer. 1955. The production and selection of a family of strains in *Penicillium chrysogenum*. *Mycologia* **47**:429-463.

Ballio, A., E. B. Chain, F. Dentice Di Acadia, M. F. Mastropietro-Cancellieri, G. Morpurgo, G. Serlupi-Crescenzi, and G. Sermonti. 1960. Incorporation of α-ε-dicarboxylic acids as side chains into the penicillin molecule. *Nature* **185**:97-99.

Barchielli, R. G. Boretti, A. DiMarco, P. Julita, A. Migliacci, A. Munghetti, and C. Spalla. 1960. Isolation and structure of a new factor of the Vitamin B_{12} group. Guanosine diphosphate factor B. *Biochem. J.* **74**:382-387.

Bradley, S. G. 1964. Genetic analysis of an unstable mutant of *Streptomyces violaceoruber*. *Develop. Ind. Microbiol.* **6**:296-301. American Institute of Biological Sciences. Washington, D. C.

Calam, C. T. 1964. The selection, improvement, and preservation of microorganisms. *Progr. Ind. Microbiol.* **5**:1-54. Gordon and Breach. Inc., London.

Darken, M. A., H. Berenson, R. J. Shirk, and N. O. Sjolander. 1960. Production of tetracycline by *Streptomyces aureofaciens* in synthetic media. *Appl. Microbiol.* **8**:46-51.

Davies, O. L. 1964. Screening for improved mutants in antibiotic research. *Biometrics* **20**: 576-591.

Dulaney, E. L., M. Ruger, and C. Hlavac. 1949. Observations on *Streptomyces griseus*. IV. Induced mutation and strain selection. *Mycologia* **41**:388-397.

Elander, R. P., J. F. Stauffer, and M. P. Backus. 1960. Antibiotic production of various species and varieties of *Emericellopsis* and *Cephalosporium*. *Antimicrobial Agents Ann.* p. 91-102. Plenum Press, Inc., New York.

Fantini, A. A. 1962. Genetics and antibiotic production of *Emericellopsis* species. *Genetics* **47**:161-177.

Florey, H. W. 1955. Antibiotic products of a versatile fungus. *Ann. Internal Med.* **43**:480-490.

Florey, H. W., E. Chain, N. G. Heatley, M. A. Jennings, A. G. Sanders, E. P. Abraham, and M. E. Florey. 1949. A survey of penicillin, streptomycin and other antimicrobial substances from fungi, actinomycetes, bacteria, and plants. p. 647-650. *In* Antibiotics. Oxford University Press, London.

Frontali, C., L. R. Hill, and L. G. Sylvestri. 1965. DNA base composition of *Streptomyces*. *J. Gen. Microbiol.*, **38**:243-250.

Haas, F. L., T. A. Puglisi, A. J. Moses, and J. Lein. 1956. Heterokaryosis as a cause of culture rundown in *Penicillium chrysogenum*. *Appl. Microbiol.* **4**:187-195.

Hopwood, D. A., and G. Sermonti. 1962. The genetics of *Streptomyces coelicolor*. *Advan. Genet.* **11**:273-342.

Huang-Lo, L. 1962. Hybridization in *Actinomyces erythreus*. *Mikrobiologiya* **31**:61-65.

Ikeda, Y., K. Nakamura, K. Uchida, and C. Ishitani. 1957. Two attempts upon improving an industrial strain of *Aspergillus oryzae* through somatic recombination and polyploidization. *J. Gen. Appl. Microbiol.* **3**:93-101. Japan.

Ishitani, C., Y. Ikeda, and K. I. Sakaguchi. 1956. Hereditary variation and genetic recombina-

tion in koji-molds (*Aspergillus oryzae* and *A. sojae*). VI. Genetic recombination in heterozygous diploids. *J. Gen. Appl. Microbiol.* **2**:401-430. Japan.
James, L. V., S. D. Rubbo, and J. F. Gardener. 1956. Isolation of high acid-yielding mutants of *Aspergillus niger* using a paper culture selection technique. *J. Gen. Microbiol.* **14**:223-227.
Jarai, M. 1961. Transformation in *Streptomyces*. *Acta Microbiol. Acad. Sci. Hung.* **8**:81-86.
Jones, L. A., and S. G. Bradley. 1963. Thermal denaturation of desoxyribonucleic acid from actinomycetes and actinophages. *Bacteriol. Proc.*, p. 148.
Karlström, O. 1965. Methods for the production of mutants as amino acid fermenting organisms. *Biotech. Bioeng.*, **7**:245-268.
Kelner, A. 1949. Studies on the genetics of antibiotic formation. I. The induction of antibiotic-forming mutants in actinomycetes. *J. Bacteriol.* **57**:73-92.
Macdonald, K. D. 1964. Preservation of the heterozygous diploid condition in industrial microorganisms. *Nature* **204**:404-405.
Macdonald, K. D., J. M. Hutchinson, and W. A. Gillett. 1963. Formation and segregation of heterozygous diploids between a wild-type strain and derivatives of high penicillin yield in *Penicillium chrysogenum*. *J. Gen. Microbiol.* **33**:385-394.
Macdonald, K. D., J. M. Hutchinson, and W. A. Gillett. 1964. Properties of heterozygous diploids between strains of *Penicillium chrysogenum* selected for high penicillin yield. *Antonie van Leeuwenhoek* **30**:209-224.
Matselyukh, B. P. 1964. Transformation of antibiotic formation among actinomycetes by means of DNA. *Dokl. Acad. Nauk SSSR* **154**:710-715.
McCormick, J. R. D., N. O. Sjolander, U. Hirsch, E. Jensen, and A. P. Doershuk. 1957. A new family of antibiotics: The demethyl tetracyclines. *J. Am. Chem. Soc.* **79**:4561-4563.
Miller, P. A., J. H. Hash, M. Lincks, and N. Bohonos. 1965. Biosynthesis of 5-Hydroxytetracycline. *Biochem. Biophys. Res. Commun.* **18**:325-331.
Mindlin, S. Z., S. I. Alikhanian, and A. V. V. Vladimirov. 1961. On a new hybrid strain of *Streptomyces rimosus*. *Sci. Rep. Ist Super. Sanita* **1**:469-470.
Nelson, T. C. 1961. Screening methods in microbiology. *Mutation and Plant Breeding Publ.* **891**:311-335. Nat. Acad. Sci., Nat. Res. Council Publ., Washington, D. C.
Orgel, L. E. 1965. The chemical basis of mutation. *Advan. Enzymol.* **27**:289-346.
Ostroukhov, A. A., and V. D. Kuznetsov. 1963. An accelerated method of selecting active variants of penicillin producers by means of rH_2-indicators. *Antibiotics (USSR)* **8**:33-35.
Pontecorvo, G. P. A., and J. A. Roper. 1958. Synthesis of strains of microorganism. *U. S. Pat.* 2,820,742.
Pontecorvo, G. P. A., J. A. Roper, and E. Forbes. 1953. Genetic recombination without sexual reproduction in *Aspergillus niger*. *J. Gen. Microbiol.* **8**:198-210.
Pontecorvo, G. P. A., J. A. Roper, and G. Sermonti. 1954. Parasexual recombination in *Penicillium chrysogenum*. *J. Gen. Microbiol.* **11**:94-101.
Raper, K. B. 1946. The development of improved penicillin-producing molds. *Ann. N. Y. Acad. Sci.* **48**:41-57.
Scherr, G. A., and M. E. Rafelson, Jr. 1962. The directed isolation of mutants producing increased amounts of metabolites. *J. Appl. Bacteriol.* **25**:187-194.
Shamoian, C. A., A. Canzanelli, and J. Melrose. 1961. Bach mutation of a *Neurospora crassa* mutant by a nucleic acid complex from the wild strain. *Biochim. Biophys. Acta* **47**:208-211.
Sermonti, G. 1961. The parasexual cycle in *Penicillium chrysogenum* and its application to the production of penicillin. *Sci. Rep. Ist Super. Sanita* **1**:449-454.
Sermonti, G., and D. A. Hopwood. 1964. Genetic recombination in *Streptomyces*. *Bacteria* **5**:223-251. Academic Press, New York.
Sermonti, G., and I. Spada-Sermonti. 1958. Genetics of *Streptomyces coelicolor*. *Ann. N. Y. Acad. Sci.* **81**:854-861.
Thom, C., and R. A. Steinberg. 1939. The chemical induction of genetic changes in fungi. *Proc. Nat. Acad. Sci. U. S.* **25**:329-335.