Genetics and Breeding of Industrial Microorganisms

Edited by

Christopher Ball

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PREFACE

A wide variety of species fall under the heading of industrial microorganisms. Not all of these have been genetically studied in depth. However, in general, the most commercially important ones have been investigated and are represented in this text. The one notable omission, *Escherichia coli*, is the most understood microorganism from a genetical point of view. This omission was prompted by two main considerations namely that the genetics of *E. coli* is extensively discussed in other texts and that its position as an organism of choice in the generation of industrial fermentation products has still to be firmly established. Finally, a provocative historical introduction is included in this book to stimulate thought about industrial/academic collaboration in genetics to solve realistic problems in industrial microbiology.

THE EDITOR

Christopher Ball, Chairman and Chief Executive Officer of Panlab Genetics, Inc., received his B.Sc. and Ph.D. degrees respectively in biochemistry in 1961 and genetics in 1965 from the University of Sheffield (U.K.).

From 1965 to 1967, he lectured in genetics at the University of Sheffield. He then worked for Glaxo (U.K.) from 1967 to 1979 in the fermentation research and development departments and was appointed head of the genetics section in 1973. In 1979 he joined E. R. Squibb (U.S.A.) in the capacity as Assistant Director of Biological Process Development and subsequently became Director of Biotechnology. In 1983 he moved to his present position with a new biotechnology company Panlab Genetics, Inc., a company devoted to furthering technology developments in industrial microbial genetics and molecular biology.

Dr. Ball has been Vice Chairman of the North-West branch of the Institute of Biology (U.K.) and co-editor of *Aspergillus Newsletter*. In 1974 he was on the organizing committee of the symposium Genetics of Industrial Microorganisms (GIM 74) and on the international advisory board of GIM 82. He is a member of the ASM and SIM (U.S.A.) and formerly the SGM and Genetical Society (U.K.).

Dr. Ball is an extensive publisher and educator in applied microbial genetics and has received many international lecturing invitations. He has also acted as Ph.D. theses supervisor and examiner in fungal genetics.

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Chapter 1

HISTORICAL INTRODUCTION

Giuseppe Sermonti

When industrial microbiology spread, following the initiation of the antibiotic era, around 1945, the genetics of fungi had already experienced a great development, due to the classic work of G. Beadle and E. Tatum with *Neurospora* and the intriguing findings of C. C. Lindegreen with Baker's yeast. Subsequently, the "American" *Neurospora* was flanked by the "British" *Aspergillus*, investigated by the group of G. Pontecorvo. In the same period, the bacterium *Escherichia coli* K 12 came on stage with the discovery of gene recombination and transduction by J. Lederberg and his co-workers in Madison, Wis. and the continuation of its *tournée* at the Institute Pasteur in Paris, where the peculiar nature of prokaryotic fertilization was clarified. The decade 1945 to 1955 was a golden age for microbial genetics and at the same time was a triumphal march of the microbial breeders, who had started the cultural and genetic improvement of the industrial strains for the production of antibiotics and other minor microbial products. The pioneers in such microbial breeding were M. Demerec at the Carnegie Institution in Washington and later M. P. Backus and J. F. Stauffer in the Botany Department of the University of Wisconsin. Although the latter worked in the same city and in the same period as J. Lederberg, they were virtually unknown to each other.

It was not by chance that the microbial species adopted by the geneticists were not the same as those "bred" by the industrial microbiologists. The "genetic" microbes were particularly suited for genetic analysis, while the "industrial" microbes were especially proficient in secreting antibiotics. The latter belonged to the genera *Penicillium* and *Streptomyces*, which received virtually no attention by the geneticists, until the very end of the golden decade 1945 to 1955.

Baker's yeast, which had been the object of both genetic and industrial research much before the last world war, was a case of its own, probably because the beer, wine, and bread products were too mundane to encourage a strong association of science and technology. Nevertheless they gave the name to the "fermentation industry", because the antibiotics had to be produced in "fermenters" similar to the beer tanks, although the process they carried out was not literally a fermentation, in the original sense of an anaerobic process.

It was just a curiosity of history that the "Genetics of Industrial Microorganisms" had to begin with the discovery of genetic recombination in *Penicillium* and *Streptomyces*. Apart from yeast, other quite respectable species, such as *Bacillus* and *Aspergillus*, had a fair genetics and were utilized for the industrial biosyntheses of important products (such as bacitracin and citric acid) long before 1955.* But the challenge was issued when *Penicillium* and *Streptomyces* joined the family of the genetically suitable microorganisms.

The genetics of these two genera were indeed started with the deliberate purpose of contributing to microbial breeding and both genera were forced to undergo recombination, although both appeared refractory to sex and both had been classified among nonsexual microorganisms. The detection of "parasexual" recombination in *Penicillium* and *Streptomyces* (in this second genus the term "parasexual" was seldom used) was a small challenge before the big challenge and it was successful. In 1970 the first International Congress on Genetics of Industrial Microorganisms was held in Prague (Czechoslovakia) and was entitled

* Both were known in 1945; for bacitracin see Johnson et al., *Science*, 102, 376, 1945; for citric acid see review by Van Loesecke, *Chem. Eng. News*, 1952, 23, 1945.

Actinomycetes and Fungi.[1] The term Genetics of Industrial Microorganisms (G.I.M.) was used by Z. Vaněk, for this meeting covering genetics (and biochemistry) of microorganisms related to industrial microorganisms, but covering little genetics of the industrial activities of the microorganisms. In the conclusion of the introductory talk, S. I. Alikhanian[2] opened the list of the perspectives of microbial hybridization as follows: "just as for higher forms, hybridization of microorganisms allows us to obtain variants with the desired genotype". The meeting was concluded by A. L. Demain cheering the fact that after so many years of engagement, there was a marriage between genetics and industry.

The occasion was exciting: one could in theory obtain, in the short span of a few years in the field of microbiology, what plant and animal breeders had painfully gained in hundreds if not thousands of years by means of hybridization and selection (cross-breeding). The "microbial breeder" had the remarkable comfort of dealing with thousands of specimens in a small space, with generation times of hours, days, or, at most, weeks, and selective devices unthinkable with plants and animals. The relevant products were not, moreover, as complex as milk, or meat, or cotton, but specific chemicals easily detectable and amenable to quantitative assay. Billions of dollars were expected to flush into the new market. In 1978, $4.2 billion in world bulk sales of antibiotics were reported.[3]

It was possibly the first time in history that biological science was called to provide defined results by means of sophisticated tools just made available. Previous "applications" of biological discoveries, as in the field of vaccines, had actually been empirical achievements only later scientifically interpreted and rationalized. Even animal and plant breeding had been for centuries more art than science and only recently, and to a limited extent, had profited of genetic rational procedures. The knowledge of chromosomes, genes, or DNA had been substantially foreign to the progress achieved by plant and animal breeders, who had at most used artificial insemination and some biometrical rationales. Empiricism had been king.

The challenge of microbial genetics represented therefore a break-point in biology. A new relationship between basic science and technology was foreseen, technology being a derivative from science. Although this relationship had been assumed several times, it had been so far illusory. Actually the history of science had defeated the expectation. "The naive picture of technology as applied science," wrote D. de Solla Price in 1969, "simply will not fit the facts. Inventions do not hang like fruits on a scientific tree . . . It is quite apparent that most technological advances derive immediately from those that precede them."[4]

Considering Genetics of Industrial Microorganisms in retrospective we can say today that the hopes were defeated from the beginning. Virtually no significant result was obtained by means of genetic recombination in either fungi or streptomycetes. This was clear in 1974, when the second G.I.M. meeting was held in Sheffield,[5] roughly 20 years after the introduction of cross-breeding in industrial microbiology. In the opening address (as an outsider) G. Pontecorvo[6] stated, "One thing is clear to the outsider: the advances in the application of genetics to the improvement of strains of industrial microorganisms are trifles compared to the advances in the fundamental genetics of microorganisms . . . The main technique used is still a prehistoric one: mutation and selection." It was during the Sheffield meeting that a Steering Commission for Microbial Breeding was first established (it had been proposed 2 years before at a Fermentation Symposium in Kyoto by Prof. H. Heslot). The term Microbial Breeding was subsequently replaced by the more prestigious Genetics of Industrial Microorganisms.

To explain the earlier poor results of the applications of microbial genetics to industry, Pontecorvo[6] suggested some organizational shortcomings, complaining the fragmentation of the work and the predominance of the chemical outlook in the microbiological industries. I would favor an interpretation, in agreement with the de Solla's statement science and technology are surely connected, but the latter is not necessarily an application of the former.

The theories of science refer to idealized and oversimplified pictures, the problems of technology face complex and intriguing situations. The transfer of the basic experience to practice is just utopian. If a useful result emerges from the scientist's lab, this is usually by chance, it is not the expected one, it is what is known as serendipic (from the three princes of Serendip who always found what they were not looking for).* The discovery of penicillin is such an example. In the case of microbial genetics, an already developed technique was brought into the fermentation factory to exhibit its ability. Initially it could only fail!

Should we conclude that the involvement of microbial genetics into the microbial breeding was misconceived? In part it was. I think it was possible that the microbial geneticist was providing the industrial microbiologist with rationales and tools that he suspected would not be profitable. In addition, the pure scientist appeared snobbish in front of his empirical colleagues and industrial management implying that it was their fault in failing to make science profitable. The involvement was, however, justified. It was the kind of the foreseen cooperation which was misdirected. The task of the geneticist should not have been that of the magician, but the one less gratifying, yet not less interesting, of analyzing and interpreting the practical results that the industrial microbiologist was obtaining empirically, with such large profusion.

The analytical approach, which the mutation-selection method was not able to provide, would have largely profited by the recombination procedures. During the progress of this kind of work, which requires the strict cooperation of geneticists, biochemists, and fermentation technologists, the ways of the empirical successes could have been traced back, procedures scrutinized and made more rational and economic, and perhaps some treasures still concealed for the princes of Serendip could have been revealed. This work has been rarely carried out, and after more than 30 years of strain screening in the fermentation industry only limited fundamental experience has been gained. If one should begin the development of a new strain for the synthesis of a new product today, one would virtually have to start again from the beginning, following the same procedures empirically introduced in the 1940s by the pioneers, including many operations which have not proved to be effective, but have just found their place in the liturgy of strain improvement.

Hopwood[7] wrote, "For a long time it was the exception rather than the rule for the science of genetics to make an appreciable contribution to the genetic programming of industrial microorganisms." In the attempt to explain this frustrating situation, I wish to refer to a misconceived idea in the "application" of the cross-breeding to strain improvement. It is that of crossing two highly producing strains, selected along divergent lines, because they carry a large number of independent positive mutations. Through recombination, these could be assembled in new assortments, thus providing new genotypes possibly crowded by an unprecedented number of positive traits, some kind of over-producing monsters. Recombination, however, did not succeed. Immediately the idealized concept of "positive" mutation turned out wrong. Each mutation selected for yield improvement is advantageous only in respect to a given complex genetic background and biochemical landscape. It may be deleterious in a different context. "The possibility that a superior strain may be affected by harmful mutations cannot be overemphasized. Such mutations trace back to diverse sources . . . Such an altered expression also results from changes in the genetic background due to the induction of other mutations . . ."[8] The primitive idea of plus-mutation[2] is hard to die, and it is still there as fundamental prejudice to jeopardize any deeper insight into the field of microbial breeding. The pretention of the scientist to teach nature how to work (and if she does not obey, she will be discounted) instead of learning from her has not saved the microbial geneticists in respect to the microbial breeding.

* The word *serendipity* was coined by Horace Walpole in 1754, after he read an oriental tale entitled "The Three Princes of Serendip", to mean: "The faculty of making happy and unexpected findings by chance", or "the assumed faculty of finding precious and pleasant things without looking for them".

In 1978, when the 3rd Symposium on Genetics of Industrial Microorganisms was held in Madison (Wis.),[9] the situation appeared to have changed. " . . . It is no exaggeration to say that a revolution has occurred in our subject in the four years since the Second International Symposium in the Genetics of Industrial Microorganisms (G.I.M. 1974)".[10] The revolution was marked by the introduction of Genetic Engineering in the fermentation industry. It is worth recalling that, during the previous G.I.M. Symposium in Sheffield (G.B.), a group of attendees had voted in a large majority in favor of the embargo decreed in the same year by a special commission of the American National Academy of Sciences to the study of recombinant DNA. However, the later perspective was to use the tools of the new genetics in the fermentation field despite the dangers (later minimized) signaled in the U.S.

Beside the in vitro recombinant DNA techniques, Hopwood[10] listed three new recombinational tools: (1) in vivo rearrangement by transposable genetic elements (1974), (2) protoplast fusion in bacteria (1976), and (3) recombination in vivo by restriction enzymes (1977). These scientific advances, he remarked "have given us the potential both to put sophisticated genetics into organisms that make useful products and to put useful products into organisms that have sophisticated genetics."

Apparently his suggestions connect pure science with practical concern by some kind of transplant, which would give us hybrids endowed with good genetics and useful products. The problem of how to use sophisticated genetics to improve industrial strain performance might not be solved in this way. One may even wonder whether the very properties which make an organism suitable to the most sophisticated genetics could not negatively affect its proficiency in the fermentor.

As a matter of fact, the effects of genetic engineering in the microbial breeding had been beneficial in many respects. First, as Hopwood[10] noted, "a significant number of the leaders in microbial genetics are now interested, directly and personally, in possible applications of their discoveries." They might not be able to replace the long experience of the practical breeder, but surely have increased the prestige of genetics in the microbial industries, thus overcoming "the predominance of the chemical outlook" complained about by Pontecorvo.[6]

A second benefit has been a fantastic refinement of the knowledge of the genetic structures and mechanisms in microbial species which would have not been otherwise object of any attention by geneticists.[11] This is rather a contribution of industrial microbiology to microbial genetics than vice versa. That things would have gone in this direction was foreseen by the present author in 1969 when he wrote: "practical problems, with their compulsory and uncomfortable roads, are a rich and irreplaceable source of raw material for basic science, which otherwise runs the risk of becoming abstract as some modern art".[12]

What about the contribution of microbial genetics to microbial breeding after the "biotechnological revolution"? I do not think that the situation has substantially changed. The analysis and the scrutiny of already empirically achieved results is still badly needed. If we continue to reason only on simple models, in terms of positive mutations to be assembled in new strains or of antibiotic-producing apparatuses to be transferred from one species to another, we will not realize the full potential of microbial genetics. It is, however, gratifying that our analytical possibilities have much improved and this promises a future insight into the mechanisms of secondary metabolism which was possibly not achievable with the traditional tools. At any rate a collaboration between biochemists and geneticists is the central point. It will not be sufficient that they speak at the same congress or publish in the same book. They have to understand each other and produce a generation of new research workers with a combined outlook in biochemistry, genetics, and also in fermentation engineering.

The most promising achievement of the recombinant DNA approach was the one that nobody would have forecast at the beginning of the 1970s: the possibility of producing animal or plant proteins in microbial fermentation. We may imagine the princes of Serendip

coming back from their adventure. As students of applied microbial genetics, they had left with the goal of obtaining improved antibiotic-producing strains and they eventually returned with an unexpected treasure: microbes which produce insulin, interferon, human growth factor, and vaccines!

It is not my task to foresee if these new microbial productions will be able to commercially compete with the same products directly produced by the higher organisms in vivo or in vitro. Their transfer into microorganisms is for sure a formidable theoretical achievement proving the universality of the genetic machinery and language from the bacterium to the man, and the availability of life to be promiscuously rearranged with no regard to the kingdoms recognized by the taxonomists among the variable expression of living matter.

Genetics of industrial microorganisms has completely changed its features. While during its first 20 years (1955 to 1975) it was made up by chromosome maps (circles in Streptomycetes and bars in fungi), biosynthetic pathways of secondary metabolites, and rising slopes showing antibiotic yield increases with the years (indeed loosely related to each other), in the present it appears so different that one can seriously question if we are still dealing with the same subject. Probably the constant trait is the leading role still retained by the genus *Streptomyces*,[13] which was able to shift from an ideal organism for sophisticated formal genetics to a quite respectable host for plasmid and virus vectors suitable to export and reimport genes and promoters from various sources.

The new look of genetics of industrial microorganisms (as apparent in G.I.M. 1982 in Kyoto) consisted of electrophoresed DNAs, restriction maps, DNA sequences, and other manifestations of molecular biology. Microorganisms which were held as venerable models (external to "industrial workers" as the filamentous streptomycetes and molds) claim their right to be accepted as bona fide industrial organisms, being able themselves to host genes coding for industrial products. Thus, *Escherichia coli* entered the family of industrial microorganisms, together with *Bacillus* and *Pseudomonas*, not for their own products, but for their gracious hospitality to foreign DNA manufacturing industrial proteins. The same applies to yeasts, the ancestors of industrial microbiology, now made available as abode for new genes imported from unrelated species.

Genetics and breeding of industrial microorganisms are again in ascension, fed with new hopes, faced with new frontiers. Can we trust in the unprecendented refinement of our tools, or do we need to reconsider the preceding history to adjust our efforts, so as to not meet again with the past disillusionment on our new path?

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Chapter 2

STREPTOMYCETES

David A. Hopwood and Keith F. Chater

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I. INTRODUCTION

Before 1955, actinomycetes were the subjects for some fundamental and applied studies of radiation genetics and mutagenesis, and a few discoveries of general significance were made (the first case of photoreactivation was a notable example);[1] however, most research on streptomycete variability was severely handicapped by the lack of a capability for genetic analysis (e.g., References 2, 3). The situation looked much brighter after the discovery, in the mid 1950s, of genetic recombination in several streptomycetes,[4-7] but these advances[8] were slow to be exploited in the area of antibiotic research, even though steady progress was made in fundamental work after the first genetic mapping procedure was developed in *Streptomyces coelicolor* A3(2)[9] and a rudimentary complementation system was discovered.[10,11] Genetic analysis became more straightforward when the linkage map was shown to be circular[12,13] and a sex plasmid was discovered which interacted with the chromosome to promote efficient recombination.[14-16] Parallel developments occurred with a few other strains.[17-22] The stage was therefore set to begin the analysis of some of the special biological properties of streptomycetes, notably morphological differentiation and antibiotic biosynthesis. Primarily by the isolation, phenotypic characterization, and genetic mapping of mutations causing interruptions in these processes, groups of genes were identified, each involved in an aspect of morphological or chemical differentiation: the formation of the aerial mycelium;[23] its processing into spores;[24-26] and the biosynthesis of methylenomycin,[27] actinorhodin,[28,29] chloramphenicol,[30] undecylprodigiosin (the "red" pigmented antibiotic of *S. coelicolor*),[31] oxytetracycline,[32] and rifamycin.[33] In none of these systems had analysis by "classical" genetic procedures been pushed anywhere near to its limit. However, it became increasingly apparent during the late 1970s that the effort required to achieve a satisfyingly penetrating description of the genetic determination of these "secondary" functions was going to be very great. Suddenly, recombinant DNA techniques offered a realistic hope of reaching this objective and it became a number one priority to develop suitable cloning techniques for streptomycetes. The steps in this undertaking — the finding of conditions for efficient DNA uptake, the physical and functional characterization of plasmid and phage genomes, and the cloning onto them of convenient selectable markers — have been described in recent reviews.[34-36] Although considerable further refinements in vectors are undoubtedly possible, currently available vectors can be used quite effectively for isolating and studying genes of interest, as we hope to show in this chapter.

While cloning has tended to dominate the field of *Streptomyces* genetics over the last few

years, other important developments have been taking place, particularly in protoplast fusion as a route to in vivo recombination;[37-41] the use of liposomes to introduce chromosomal and other DNA into the cells;[42,43] the characterization of a variety of sex plasmids that promote in vivo recombination;[44-46] the discovery of novel kinds of plasmids, notably linear DNA molecules;[47,48] studies of genetic instability;[49,50] and the discovery of reiterated DNA of unknown function but remarkable amplification in some strains.[51-55]

In this chapter we attempt to cover the broad field of *Streptomyces* genetics, which we reviewed in a previous article initially written some 2 years before this one.[56] A comparison of the two reviews may therefore reveal the rate of recent progress of research in this area as we perceive it. The reader is also referred to other reviews[57,58] with somewhat different perspectives and a more applied stance.

II. MUTAGENESIS AND RELATED MATTERS

A. Isolation of Mutant Clones

Streptomycetes were chosen for early studies of mutagenesis because of their characteristically copious production of spores which, in general, showed radiation kinetics typical of haploid genomes; *Streptomyces griseoflavus* was an exception, with two-hit kinetics, suggesting duality of the genetic material in the spores;[59] this interesting situation appears not to have been reexamined (but see Reference 60). Haploid spores are the obvious choice for mutagenesis, but some strains, especially industrial cultures, produce them sparsely; mycelial fragments[39] or protoplasts from mutagenized mycelium[61] may be used as alternatives.

B. Quantitative Studies of Mutagenesis

Some of the few early quantitative studies of mutagenesis in *Streptomyces* are cited by Clarke and Hopwood,[62] who made the observation that mutation in *S. coelicolor* A3(2) (as judged by "reversion" of auxotrophy to prototrophy, primarily by suppressor mutations) follows two-hit kinetics. Perhaps this implies the existence of an inducible, error-prone repair system.

C. Two-Way Mutation Systems

Classically, the ability to select both forward and reverse mutations in the same gene(s) has been sought after by microbial geneticists because it offers unique opportunities for the study of the base changes involved in mutagenesis. Such systems have hitherto been lacking in *Streptomyces*. However, recent work on glucose utilization by *S. coelicolor* A3(2)[63,64] has changed the situation. Forward mutations leading to loss of glucose kinase can be selected on the basis of resistance to 2-deoxy-D-glucose, while reverse events are selected on a medium with glucose as sole carbon source. An alternative system, with selection for loss of galactose kinase on deoxy-D-galactose and reverse selection on galactose is under investigation.[65,66] Another useful forward mutation system is provided by the glycerol utilization operon (see below); a mutant lacking glycerol-3-phosphate dehydrogenase dies on a medium containing glycerol, presumably through the toxic effects of an accumulation of glycerol-3-phosphate produced from glycerol by the action of glycerol kinase, so that mutations in the kinase gene can be selected on glycerol, with a sugar such as arabinose as alternative carbon source (glucose cannot be used since it represses the glycerol operon).[64]

D. Radiation-Sensitive Mutants

Most of the UV-sensitive (*uvs*) mutations of *S. coelicolor*, which represented six loci,[67,68] were probably equivalent to the *uvr* (excision-repair) mutations of *Escherichia coli*, but mutations in two loci, *uvsE* and *uvsF*, had different survival kinetics.[69] *uvsE* mutations merely lacked the shoulder of the wild-type survival curve, while a *uvsF* mutation enhanced

the UV-sensitivity of strains carrying *uvsA, C,* or *D* mutations, but not of strains carrying a *uvsF* mutation alone. None of the mutations, when present in both parents in a mating (carrying, as we now know, the SCP1 and SCP2 plasmids and the SLP1 and SLP4 elements: see below), had a significant effect on genetic recombination.[69] In *S. fradiae*, a mutant was described with sensitivity to UV, ionizing radiations, and chemical mutagens very similar to those of *recA* mutants of *E. coli*,[70] but its recombination phenotype has not been described. It is worth pointing out that the range of systems that might be used to characterize the effect of potential *rec* mutations on recombination is now greater than at the time of the earlier studies,[69] when conjugation in strains uncharacterized in respect of sex plasmids was the only system available. We now have strains of *Streptomyces lividans* (see Section IV.A.7) devoid of endogenous sex factors which can be induced to undergo recombination by conjugation when any one of half a dozen different sex plasmids is introduced into them;[46] thus the chance that the plasmid used to promote conjugation might itself encode a recombination system that could suppress a *rec* mutation can be minimized. Another alternative is protoplast fusion (see below), which can lead to recombination at such a high frequency as to provide a very sensitive test for recombination proficiency. Tests using either matings or fusions each require the potential *rec* mutation to be homozygous and so to have been introduced into two differently marked parental strains. In contrast, recombination between differentially marked plasmid copies can be observed in a single host strain;[45] so can recombination between a cloned fragment of a wild-type gene introduced on a plasmid and the chromosome bearing a mutation, to restore the wild-type phenotype,[71] or between homologous sequences present in the chromosome and in an *att*-deleted phage carrying a resistance gene, giving drug-resistant lysogens.[72,73]

Saunders et al.[74] and Saunders and Holt[75] reported the isolation of UV-sensitive mutants of *S. clavuligerus*. They described some of the mutants, which were presumably excision-defective, as well as some of the *S. coelicolor uvs* mutants of Harold and Hopwood,[69] as "hypermutable" because they showed an increased mutation frequency compared with the wild-type, at a given UV dose. But of course the survival of the mutants was much lower than that of the wild-type under these conditions and, if one tries to compare mutation frequencies at the same survival level, mutant and wild-type have rather similar frequencies, with the mutant showing a value perhaps twice that of the wild-type. In other words, the strain is not hypermutable to any marked extent. (A similar criticism applies to the statement[75] that the wild-type *S. clavuligerus* strain showed a 3000% [i.e., 30-fold] increase in mutation frequency by UV in the presence of caffeine; this very largely disappears when comparisons are made at the same survival level.)

E. Localized Mutagenesis

Several possibilities have been suggested for localized mutagenesis in *Streptomyces*, including NTG treatment of mutagenized synchronized cultures[76] and comutation, when reversion of an auxotrophic mutation by NTG should be associated with forward mutations in nearby genes,[77] but neither has proved useful, at least to judge by published work. The former is likely to be difficult to apply with any precision and the latter suffers from the disadvantage that only a minority of the existing auxotrophic mutations in *Streptomyces* revert at high frequency with NTG.[78] Treatment by hydroxylamine of the temperate transducing phage øSV1 of *S. venezuelae* leads to localized mutagenesis,[79] but the general utility of this technique is limited by the current paucity of transduction systems (see below). The mutagenesis of heavily irradiated protoplasts, followed by their fusion to unirradiated protoplasts carrying a counterselectable marker,[80] might well work. However, now that chromosomal DNA fragments can be delivered to protoplasts with high frequency,[42] the advantages of in vitro mutagenesis of naked DNA are likely to make this a superior approach. Even more versatile, however, will be in vitro mutagenesis, including site-specific mutagenesis,