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Fresh Approaches to Antibiotic Production [and Discussion]

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Phil. Trans. R. Soc. Lond. B 1980 **290**, 313-328
doi: 10.1098/rstb.1980.0097

Phil. Trans. R. Soc. Lond. B **290**, 313–328 (1980) [313]
Printed in Great Britain

Fresh approaches to antibiotic production

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New antibiotics are needed, (*a*) to control diseases that are refractory to existing ones either because of intrinsic or acquired drug resistance of the pathogen or because inhibition of the disease is difficult, at present, without damaging the host (fungal and viral diseases, and tumours), (*b*) for the control of plant pathogens and of invertebrates such as helminths, insects, etc., and (*c*) for growth promotion in intensive farming. Numerous new antibiotics are still being obtained from wild microbes, especially actinomycetes. Chemical modification of existing compounds has also had notable success. Here we explore the uses, actual and potential, of genetics to generate new antibiotics and to satisfy the ever-present need to increase yield.

Yield improvement has depended in the past on mutation and selection, combined with optimization of fermentation conditions. Progress would be greatly accelerated by screening random recombinants between divergent high-yielding strains. Strain improvement may also be possible by the introduction of extra copies of genes of which the products are rate-limiting, or of genes conferring beneficial growth characteristics.

Although new antibiotics can be generated by mutation, either through disturbing known biosyntheses or by activating 'silent' genes, we see more promise in interspecific recombination between strains producing different secondary metabolites, generating producers of 'hybrid' antibiotics. As with proposals for yield improvement, there are two major strategies for obtaining interesting recombinants of this kind: random recombination between appropriate strains, or the deliberate movement of particular biosynthetic abilities between strains.

The development of protoplast technology in actinomycetes, fungi and bacilli has been instrumental in bringing these idealized strategies to the horizon. Protoplasts of the same or different species can be induced to fuse by polyethylene glycol. At least in intraspecific fusion of streptomycetes, random and high frequency recombination follows. Protoplasts can also be used as recipients for isolated DNA, again in the presence of polyethylene glycol, so that the deliberate introduction of particular genes into production strains can be realistically envisaged. Various kinds of DNA cloning vectors are being developed to this end.

Gene cloning techniques also offer rich possibilities for the analysis of the genetic control of antibiotic biosynthesis, knowledge of which is, at present, minimal. The information that should soon accrue can be expected to have profound effects on the application of genetics to industrial microbiology.

1. Introduction

Bérdy (1974) arbitrarily divided the history of antibiotic research into three periods: an initial slow phase up to the early 1940s, which culminated in the first therapeutic uses of antibiotics; a second, 'frenzied' period of 15–20 years up to 1960, in which new antibiotics to meet many of the major requirements for antibacterial chemotherapy were found comparatively easily; and a third phase of 'diminished returns' as far as natural antibiotics were concerned, in which various new approaches were needed to allow companies to remain significantly involved in antibiotic production. Some of the most important of these new approaches were: a greatly increased commitment to semi-synthetic antibiotics; extensive studies of the biochemistry, pharmacology and chemistry of known antibiotics, probably largely with a view to predicting

structure–activity relations helpful in the development of new semi-synthetic compounds; attempts to broaden both the basis of screening for antibiotic activities and the range of microbial genera screened; and the development of new applications for antibiotics, both within, and more especially outside, their traditional uses in human and veterinary medicine.

We suggest that a fourth phase is now beginning in which genetic techniques will increasingly be used both to generate new antibiotics and to increase the yields of existing antibiotics. This will be the underlying assumption of the present article. We shall first attempt to put these 'new horizons' in context by examining the need for genetical approaches as part of the general strategy of antibiotic production, before reviewing the progress that has been made in developing the necessary genetic methodology in antibiotic-producing microorganisms.

TABLE 1. THE SOURCES OF ANTIBIOTICS

group	total antibiotics†	genus	commercial antibiotics‡
fungi	768	*Penicillium*	3
		Cephalosporium	1
		Aspergillus	1
		Helminthosporium	1
		Fusidium	1
		Paecilomyces	1
eubacteria	372	*Bacillus*	6
		Sorangium	1
actinomycetes	2078	*Streptomyces*	69
		Micromonospora	4
		Nocardia	1

† From Bérdy (1974). ‡ From Perlman (1977).

2. THE SOURCES OF ANTIBIOTICS

Although a variety of multicellular eukaryotes are known to synthesize antimicrobial compounds, the vast majority of antibiotics, and all those at present produced commercially, are made by microorganisms: the prokaryotic eubacteria and actinomycetes and the eukaryotic filamentous fungi. The numbers of antibiotics credited by Bérdy (1974) to each of these major groups of microbes are shown in table 1. These numbers have certainly increased significantly since Bérdy's compilation, but the relative importance of the organisms has probably not changed. Also listed in table 1 are the numbers of antibiotics produced commercially by genera of microbes within these groups, taken from Perlman (1977). We see that actinomycetes, and, notably, streptomycetes are far and away the most important antibiotic producers when judged on the basis of the number of directly useful fermentation products. However, the commercial significance of fungi is greatly under-estimated by these numbers because of the pre-eminence, as broad-spectrum antibiotics, of the semi-synthetic β-lactams manufactured from penicillin G and cephalosporin C by side-chain substitution. Probably something over half the world sales of therapeutic antibiotics are accounted for by these indirect fungal products, while the great majority of the remaining sales are of actinomycete products, including some of major therapeutic importance (tetracyclines, erythromycin, chloramphenicol, lincomycin and various aminoglycosides), and a larger number with more specialized therapeutic markets (rifamycin, novobiocin, amphotericin B, etc.). The actinomycetes are certainly pre-eminent as producers

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of non-therapeutic antibiotics, notably those used as animal feed additives (e.g. monensin and tylosin) or as agents for the control of plant diseases (e.g. blastocidin S, kasugamycin and polyoxin). By comparison, no eubacterial antibiotic is of major quantitative significance, although a few (the oligopeptide antibiotics like bacitracin, polymyxin and tyrocidine) are useful in special situations.

What moral ought we to draw for the future from a consideration of the present sources of antibiotics? While invaluable new compounds might await discovery in groups of organisms that do not yet contribute any, more favourable odds must surely apply to the filamentous fungi and actinomycetes, and more particularly to the latter because of the extreme chemical diversity of their antibiotic products, which even include a variety of β-lactam nuclei (see §4*b*), until recently thought to be confined to the fungi, as well as oligopeptide antibiotics (such as capreomycin and viomycin), a chemical class characteristic of bacilli.

TABLE 2. SOME NEEDS FOR NEW ANTIBIOTICS

(*a*) new antibacterial agents
  (i) to attack intrinsically insensitive bacteria
  (ii) to overcome acquired resistance
  (iii) to improve pharmacological properties
(*b*) other medical applications
  (i) less toxic antifungal antibiotics
  (ii) less toxic antitumour antibiotics
  (iii) antiviral antibiotics
(*c*) growth promotion without cross resistance
(*d*) control of plant pathogens, helminths, coccidia, insects, etc.

3. NEEDS FOR NEW ANTIBIOTICS

Are new antibiotics really needed? This would have been a naïve question in the early 1940s, when the only antibiotic available was the first naturally produced penicillin, which was highly unstable, could not be administered orally, and was effective only against Gram-positive bacteria. However, the situation is so different today that it is legitimate to ask whether the antibiotic *armamentarium* is now sufficiently comprehensive.

In table 2, we list some reasons why new antibiotics should be developed. To varying degrees these requirements (except for anti-viral compounds) are already being met, but there is considerable scope for improvement under all the headings listed.

(*a*) *New antibacterial agents*

Antibiotics succeed as selective antibacterial agents in chemotherapy because prokaryotes differ from eukaryotes in crucial aspects of their biochemistry; thus, sensitive targets, such as the bacterial RNA polymerase, ribosome or cell wall, can be attacked without killing animal cells. However, certain groups of bacteria are hard to eliminate with antibiotics.

In general, Gram-negative organisms, because of their extra 'outer membrane', are considerably less sensitive than Gram-positive species and, in spite of the significant advances made in the development of broad-spectrum semi-synthetic β-lactam antibiotics, it is probably still true to say that ideal antibiotics for the control of several members of genera such as *Pseudomonas*, *Serratia* and *Proteus* are not available. Demain (1977) has suggested the development of new antibiotics that would selectively inhibit outer membrane synthesis for use as chemotherapeutic agents in combination with other antibiotics to which Gram-negative organisms are ordinarily

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too insensitive for effective chemotherapy. Another genus of refractory pathogens is *Mycobacterium*. In this case the main problem appears to be the very slow growth rate of the bacteria within infected tissues. Thus chemotherapeutic treatment of tuberculosis and leprosy (still enormously important diseases in the Third World) is long, expensive, and only partially effective. Ideal antibiotics against these diseases are not yet available.

Many antibiotics that were entirely satisfactory in the treatment of bacterial infections are no longer effective in their original context because the pathogens have become resistant, usually by the acquisition of resistance determinants on transmissible plasmids from other, non-pathogenic, bacteria (Falkow 1975). The recent origin and spread of penicillin-resistant gonococci gives particular cause for concern (Elwell *et al.* 1977). Thus, the development of new antibiotics has to continue even to maintain, let alone enlarge, the present ability to treat infectious bacterial diseases. Probably, the spread of drug resistance could be significantly retarded by reduction of the abuse of antibiotics (by restricting therapeutic antibiotics to therapeutic uses, and by more rational prescription of antibiotics), but at some level the problem will always remain and will provide a continuing requirement for new antibiotics, effective precisely because of their novelty.

Although antibacterial antibiotics are always, of necessity, more toxic to the target bacteria than to their eukaryotic hosts, few antibiotics are completely non-toxic to mammals at the doses required to eradicate bacterial pathogens. Thus, antibiotics with lower toxicity will always be useful. Other important pharmacological properties include stability to acid in the stomach, and retention time in the body, and these also are usually capable of improvement.

(*b*) *Better antifungal, antitumour and antiviral antibiotics*

Since fungi causing human and animal disease are eukaryotic parasites within eukaryotic hosts, they are hard to kill selectively. Thus, existing antifungal antibiotics of streptomycete origin (e.g. amphotericin B, nystatin and candicidin) are highly toxic and leave much to be desired as chemotherapeutic agents, except for topical applications. It is not impossible that fungi have biochemical peculiarities that could be exploited in selective chemotherapy. Who would have predicted the significant metabolic differences among angiosperms that have allowed the development of selective herbicides active against only some members of the group? So a search for more suitable antifungal antibiotics is certainly warranted.

This problem arises even more poignantly in attempts to kill cancer cells selectively, without extensive damage to normal tissues. Hence existing antitumour drugs, including the several important streptomycete antibiotics (bleomycin, daunomycins, actinomycin D, etc.), have serious side effects, and it remains to be seen whether it will be possible to find antibiotics more selectively active against neoplastic cells.

In spite of much effort spent in screening, there appears to be no antibiotic (or other chemotherapeutic agent) that satisfactorily inhibits virus development without interfering with the central molecular biological processes of the host cell on which virus multiplication depends. Of course, this does not mean that continued attempts to find antiviral antibiotics are not justified.

(*c*) *Growth promotion without cross resistance*

There is an enormous demand for antibiotics which, when added to animal feeds, modify the gut flora to increase significantly the efficiency of food conversion. Unfortunately, the use of therapeutic antibiotics in such applications exerts extra selective pressure for resistant

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organisms, which may eventually hasten the build up of resistance in pathogenic bacteria. Ideally, antibiotics marketed as feed additives should not resemble chemically antibiotics used in human and veterinary medicine sufficiently to give rise to cross resistance to such antibiotics. The great chemical diversity of actinomycete antibiotics is invaluable here, since it allows the allocation to growth promotion of antibiotics, such as monensin (Stark 1977), that appear to be well suited for this agricultural market. There is considerable scope for the introduction of further antibiotics as growth promotants, partly because the desirable attributes of such compounds differ when used in different animals (ruminants, non-ruminant mammals or birds) and partly to ensure a market share for each company with a legitimate interest in this area.

(*d*) *Control of plant pathogens and invertebrates*

There are a number of important agricultural applications of antibiotics besides their use as feed additives, and these could be expanded considerably by the introduction of new antibiotics. Experience in Japan has shown the usefulness of antibiotics in the control of plant diseases, such as those of rice, by blasticidin S (Perlman 1968), or of some fungal orchard diseases, by polyoxin (Hori *et al.* 1974). As far as invertebrate parasites are concerned, diseases caused by helminths and coccidia are already treated with antibiotics (Bérdy 1974), but other possible applications exist, for example, the control of infestations by insects and mites (H. Zähner, personal communication).

4. Production of new antibiotics

(*a*) *Chemical modifications of existing antibiotics*

The semi-synthetic approach has produced some extremely valuable antibiotics, but the success rate is strikingly low. Thus Bérdy (1974) estimated that nearly 30 000 chemical derivatives of natural antibiotics had been prepared, of which fewer than 50 had been used clinically. The average was depressed somewhat by penicillin derivatives (23 out of 20 000, or 0.1%, were used clinically), the success rate for other classes of antibiotics (derivatives of cephalosporin C, tetracyclines, rifamycin, kanamycin, chloramphenicol, lincomycin and streptomycin) being 24 out of 9800 (0.25%).

Semi-synthetic modification is potentially applicable to each of the requirements for new antibiotics listed in table 2, with the probable exception of (*c*), but its proven success is essentially confined to the antibacterial field. Thus, semi-synthetic modification of penicillins and cephalosporins has conferred significant Gram-negative activity on antibiotics that otherwise lack it; the same approach has resulted in β-lactam antibiotics capable of resisting the β-lactamases of pathogenic bacteria that have acquired resistance, and in oral penicillins and cephalosporins resistant to the low pH of the stomach. While the β-lactams dominate the semi-synthetic field, other antibiotic classes are also represented. For example, some modified kanamycins have become resistant to enzymic modification by certain resistant organisms, and doxycycline has a distinct advantage, in its increased retention time in the body, over the natural oxytetracycline.

Probably, the semi-synthetic approach will continue to be very valuable in the development of new antibiotics, but it is unlikely to provide for all future requirements any more than it has in the past; the discovery of new classes of antibiotics, susceptible, perhaps, in their turn to improvement by chemical modification, will also be needed.

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(*b*) *Screening wild isolates*

The traditional way of discovering new antibiotics, by the examination of newly isolated microorganisms from natural sources, usually soil samples, continues to reveal new antibiotics, and at a rate that shows little evidence of decline. The total number of new antibiotics described per year increased at an approximately linear rate from the early 1940s to at least the early 1970s, when it was running at about 200 annually, of which about 130 came from actinomycetes (Bérdy 1974); and actinomycetes alone are still contributing at least 150 new antibiotics per year (see supplements to *Index of antibiotics from actinomycetes* in each issue of *The Journal of Antibiotics*). In contrast, the rate of discovery of new antibiotics that have found practical applications has declined sharply since 1960. Obviously, it has become more difficult to find new antibiotics either to serve new applications when the 'easier' applications have been increasingly satisfied, or sufficiently superior to existing compounds in their present applications to warrant the escalating cost of marketing a useful, but not outstanding, new product.

In spite of these considerations, there are some promising new compounds close to commercialization. Probably the most significant are the β-lactam antibiotics of actinomycetes, including the cephamycins and thienamycin, which were discovered quite recently (Hamilton-Miller & Brumfitt 1975; Tally *et al.* 1978). Another exciting development is that of β-lactamase inhibitors, such as clavulanic acid, also from streptomycetes (Reading & Cole 1977; Neu & Fu 1978). These compounds are themselves weak antibiotics but, when administered together with a penicillin or cephalosporin that is sensitive to a β-lactamase, should overcome the resistance conferred by that enzyme (see, for example, Miller *et al.* 1978).

Awareness of the inevitable diminishing returns that accompany the continuing isolation and screening of familiar types of soil microorganisms by traditional methods has stimulated the widening of screening methods (Hamil 1977) and the selective isolation of organisms from unusual habitats, where different ecological requirements may have produced a different spectrum of antibiotics from those found in soil organisms readily isolated on the usual laboratory media; hence the current interest in members of 'novel' actinomycete genera, such as *Actinoplanes*, *Streptosporangium*, *Geodermatophilus*, etc. (Nara *et al.* 1977).

(*c*) *Genetic approaches*

Both mutation and recombination can undoubtedly give rise to useful new antibiotics even if the precise routes for achieving this goal are still somewhat speculative. A well known example of mutation leading to production of a different member of a chemical family of antibiotics is the loss, by a mutant of *Streptomyces aureofaciens*, of the ability to chlorinate the tetracycline nucleus in the 7-position, leading to accumulation of tetracycline instead of chlortetracycline. A more radical possibility is the mutational activation of 'silent' genes, on the hypothesis that individual actinomycetes have the genetic potential to synthesize a greater variety of antibiotics than they normally produce.

An example of a mutation leading to detection of a new antibiotic is the production of an orange pigmented antibiotic by a mutant of *Streptomyces coelicolor* A3(2) (Rudd 1978), the original culture of which normally synthesizes two other pigmented antibiotics (actinorhodin and a red compound of unknown structure), as well as the colourless methylenomycin A. The mutation (*ora*) leading to detectable expression of orange pigmentation was mapped to the chromosome, in a position quite distinct from the clusters of structural genes controlling the other two pigmented antibiotics.

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Renaturation kinetics has shown that streptomycetes contain a larger complement of DNA than many other prokaryotes (some 2–3 times that of *E. coli* or of *Bacillus* species: Antonov *et al.* 1978). Since there is no evidence that this 'extra' DNA is accounted for by a significant proportion of repetitive sequences, what is its function? It seems unlikely that it represents genes specifically involved in differentiation, since the number of genes recognizable by mutations interrupting normal development is small, of the order of a few tens (Chater & Merrick 1979), and, in any case, the endospores of bacilli are probably rather more complex in their genetic control than the aerial spores of streptomycetes (Piggott & Coote 1976). Thus, a considerable amount of normally unexpressed genetic information for antibiotic synthesis could conceivably exist.

We suggest that a suitable protocol for the screening of wild isolates for new antibiotic activities after mutagenesis would reveal some interesting compounds production of which at detectable levels under normal conditions was not to the selective advantage of wild isolates. (It may be significant that the *ora* mutants of Rudd (1978) grow less vigorously than *ora*$^+$ strains and that the orange compound secreted by *ora* mutants shows antibiotic activity against isogenic *ora*$^+$ strains when they are at a metabolic disadvantage, for example, when their growth is limited by shortage of an essential nutrient.)

Little is known about the genetic regulation of antibiotic biosynthesis (see §5*d*), but it is likely *a priori* that structural gene expression is controlled by regulator genes distinct from the structural genes. Thus, as an alternative to mutation, random recombination between different wild isolates might be expected to reveal new antibiotic activity by separating structural genes from 'over-efficient' repression or by the removal of metabolic interference (both methylenomycin A and the orange antibiotic in *S. coelicolor* A3(2) interfere with production of actinorhodin and the red antibiotic).

A more certain and more rational application of recombination to the generation of new antibiotics is the transfer of genes between strains or species making different members of the same chemical family of antibiotics, to produce a strain making a 'hybrid' molecule. There is considerable evidence that the enzymes of secondary metabolism are less specific than those of primary biosynthetic pathways (Hopwood & Merrick 1977) and a case in point is the production of new antibiotics (usually aminoglycosides in present examples) by the technique of 'mutational biosynthesis' (Nagaoka & Demain 1975) or 'mutasynthesis' (Rinehart 1977; Shier *et al.* 1969). Mutants blocked in the synthesis of the aminocyclitol moiety of such antibiotics are fed with analogues of the normal moiety, which are accepted by the biosynthetic enzymes, to produce novel aminoglycoside antibiotics. This suggests that enzymes from two different biosynthetic pathways could cooperate to produce such novel compounds if the appropriate recombinant organism were made.

The first attempts to put this idea into practice would logically be made with pairs of strains making different aminoglycosides, macrolides, β-lactams, etc., but, in principle, there is no barrier to the formation of a hybrid between different chemical classes of antibiotics. A new antibiotic might also result from the intervention in an antibiotic pathway of a foreign non-specific methylase, hydroxylase, dehydrogenase, etc. not associated with antibiotic production in the donor organism. An example of a 'wide' chemical hybridization may in fact already have occurred, although the published evidence is incomplete. Fleck (1979) reported that an interspecific hybrid of a *Streptomyces hygroscopicus* mutant blocked in the synthesis of the macrolide turimycin and a *Streptomyces violaceus* mutant blocked in the synthesis of the (glycosylated)

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anthracycline antibiotic violamycin produced a new antibiotic (iremycin). This contains an anthracycline nucleus, but differs from violamycin in the position of a sugar residue. The evidence that the *S. hygroscopicus* parent contributed structural genetic information to the new molecule is slender; perhaps we are seeing an example of an altered expression of genetic information by recombination involving regulatory genes of the type suggested above. Whatever the mechanism, however, this example is interesting in emphasizing the validity of a recombinational approach to the discovery of new antibiotics. It also emphasizes the need for information on the number and organization of structural genes for antibiotic biosynthesis, a subject on which very little information still exists, if new hybrid antibiotics are to be constructed rationally and if the origin of putative chemical hybrids is to be correctly interpreted (Hopwood & Merrick 1977; Hopwood 1978; Chater 1979).

5. Increasing antibiotic yields

(*a*) *Mutation and selection*

Antibiotics are typically produced by wild microorganisms in their natural environment at very low levels. To manufacture them commercially, and perhaps even to produce enough material for structural determination and the evaluation of pharmacological properties, etc., the yield has to be increased. The traditional and, to date, principal method of yield improvement has been the isolation of improved genotypes by random mutation and screening, coupled with attempts to optimize fermentation conditions, including the addition of specific nutrients appropriate to particular biosyntheses. This approach has resulted in yield increases of some of the major antibiotics to remarkable levels (for example, at least 20 g/l for penicillin, and in excess of 30 g/l for some streptomycete products), but enormous amounts of labour and materials have been required to develop the strains. Yield improvement programmes, moreover, have progressed at an unavoidably slow rate, limited by the real time needed for each round of mutation, screening and evaluation; this follows from the nature of the genetic control of antibiotic yield, which behaves as a typical quantitative character controlled by very large numbers of genes, many playing a comparatively minor role. Evidently, genes having an indirect effect on yield, by affecting the supply of primary metabolites to antibiotic pathways, the diversion of antibiotic precursors from such pathways, the concentrations of cofactors, the availability and channelling of energy, the operation of permeability barriers, the tolerance and export of antibiotics by the cell, etc., far outnumber the couple of dozen genes directly involved in specifying the biosynthetic enzymes of even the longest antibiotic pathways themselves. Empirical observations that yield improvement pedigrees usually progress by many small sequential steps, rather than by a few large increments, have been complemented by biometrical analysis indicating several hundred or a thousand genes influencing penicillin yield in *Aspergillus nidulans* (Simpson & Caten 1979).

Probably, the only 'new horizon' affecting the traditional approach to yield improvement has been the use, either by a contract company, such as Cetus Corporation, or by the pharmaceutical companies themselves, of semi-automated facilities capable of screening large numbers of isolates; these companies aim to handle tens of thousands rather than hundreds of survivors of mutagenesis in each round of the selection programme. Such systems increase the chance of finding the highest yielding genotypes available at each round of selection, but the time interval between successive rounds is still critical in limiting progress. Moreover, because of the notorious

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difficulty of correlating the performance of different genotypes under small scale screening conditions with those in large scale production (merely an example of the general rule that quantitative characters are controlled by a combination of genetic and non-genetic factors and their interactions), the design of miniature screening facilities is critical for each organism, product and fermentation plant, while subjective decisions have constantly to be taken as to when, and to what extent, fermentation parameters on the plant should be changed to optimize the yield of a promising new genotype.

Against this background, we consider the role that recombination can play in yield improvement.

(*b*) *Recombination of divergent lines and wild-types*

Since the aim in yield improvement is the accumulation, in one individual, of as many as possible of the potential yield-enhancing alleles, recombination between divergent selection lines in a strain improvement pedigree, or between different wild-types or their derivatives, should greatly shorten the real time needed to reach a particular goal in antibiotic titre, provided that the various genes from the different lines interact in a favourable way. Hopwood (1979) gave an example of an idealized strain selection pedigree in which two divergent lines of common origin were each improved by five rounds of mutation and selection before being recombined. On the reasonable assumption that all ten mutations that had been selected, five in each line, were different, potentially 2^{10} (1024) different genotypes would occur among the recombinant progeny. The generation of a markedly superior strain by this single round of recombination would depend on the mode of interaction of the ten pairs of alleles. If all interactions were additive or multiplicative, one round of recombination would be equivalent to five rounds of mutagenesis; and unless nearly all interactions were epistatic (a recombinant carrying two mutations, one from each parent, having the yield of one of the parents), some advantage would accrue from recombination.

Additive, multiplicative and epistatic interactions all occur in various situations. Thus, the natural variation in penicillin titre among different wild isolates of the fungus *Aspergillus nidulans* was largely additive (Merrick & Caten 1975), so that, by successive recombination between pairs of wild-types, and between the selection lines thus produced, the effects of all the yield-enhancing genes could be accumulated in a single strain (Merrick 1975*a*, *b*). On the other hand, mainly epistatic interactions were seen when two divergent lines containing *induced* mutations for yield enhancement were crossed (I. N. Simpson & C. E. Caten, personal communication), agreeing with an earlier study of three such mutations (Ditchburn *et al.* 1976). A more encouraging result was described by Hamlyn & Ball (1979), involving cephalosporin C titre in the fungus *Acremonium chrysogenum* (*Cephalosporium acremonium*). Among a rather small sample (about 600) of recombinants from a protoplast fusion of two divergent lines containing induced mutations, one had an antibiotic yield some 40% greater than that of the higher-yielding parent. Further biometrical studies of antibiotic yield are clearly needed to gain insight into the best combinations of mutation and recombination in strain development; this is particularly true of streptomycetes, in which the availability of generalized recombination through protoplast fusion (see § 6*a*) should now make informative studies possible.

Useful recombination of divergent lines or wild-types may involve genes other than those concerned with yield itself. Thus, crossing independently highly developed strains with a progenitor or with each other should eliminate mutations adversely affecting growth or other cultural characteristics, which had been introduced inadvertently in the selection programme.

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(c) *Increased gene dosage*

Since the intracellular concentration of individual gene products tends to increase directly with gene dosage, gene multiplication is an obvious strategy for increasing the yield of valuable proteins or of peptides produced by template synthesis, and dramatic results have been achieved when multiple copies of genes have been generated by *in vivo* or *in vitro* genetic manipulation (Brammar 1976; Panasenko *et al.* 1977). Although increase of the dosage of the genes for antibiotic synthesis might, in principle, increase production, certain mechanistic difficulties tend to obstruct this aim. True antibiotics are made by stepwise synthesis (Hopwood 1978) involving perhaps 10–30 gene products. If the genes are scattered, it is necessary to identify the one of which the product is rate limiting before amplification can be rationally attempted. If the genes are clustered, as, in prokaryotes, many are likely to be, then a relatively large segment of DNA would have to be amplified. Moreover, increased gene dosage can only increase yield when the products of those genes are rate limiting for the total biosynthesis. According to Kacser & Burns (1973), this situation does not generally prevail for biosynthetic enzymes in wild strains. For all this, there will be some cases, potentially in all yield improvement programmes at particular stages, when biosynthetic gene products are 'limiting'; thus, duplication of an entire set of biosynthetic genes, for a whole antibiotic or a moiety or side chain of it, would significantly enhance yield.

(d) *Deregulation of antibiotic pathways*

Antibiotic production is undoubtedly closely regulated in wild-type organisms, but the mechanisms of regulation have not yet been elucidated. Some regulation is likely to be pathway specific, although no example of a specific regulator gene has yet been seriously sought. Other control mechanisms are more generalized. These include many examples of carbon catabolite repression (Martin 1978) and of inhibition by inorganic phosphate (Martin 1977), and some of nitrogen catabolite repression (Aharonowitz 1979).

None of these regulatory systems has been subjected to the kind of genetic analysis needed to allow the rational deregulation of antibiotic biosynthesis. Undoubtedly, certain yield increases in empirical strain selection have involved regulatory mutations (Demain 1972), but the ability to construct a genotype incorporating mutations in several different types of control systems should be very valuable. An interesting analogy is provided by the work of Tribe (1978), who set out to maximize the yield of tryptophan by *E. coli* K12 by the successive elimination of control bottlenecks, and achieved a remarkable yield.

In vitro genetic engineering raises the possibility of deregulation through the by-passing of control mechanisms that limit the transcription of genes for antibiotic synthesis if the genes can be attached to very efficient promoters. High transcription levels of various genes cloned into *E. coli* have been achieved in this way (see, for example, Itakura *et al.* 1977). This approach might provide an alternative to increasing gene dosage, but is evidently subject to caveats similar to those discussed in that context (see §5*c*).

(e) *The cost of yield*

Yield must be judged not only by the amount of antibiotic produced in a fermenter, but also in terms of the cost of carrying out the fermentation. For example, the ability of a strain to grow on, and produce antibiotic from, a cheaper carbon source may make it commercially more productive than a higher yielding strain requiring a special carbon source to optimize yield.

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Utilization of cheap medium may often depend on the presence in a strain of a very small number of catabolic genes, the introduction of which into a production strain might conceivably be brought about by conventional matings or protoplast fusion with a different wild-type naturally possessing the required metabolic capability. More attractively, the goal might be reached by gene cloning techniques, which are ideally suited to the implantation, in a new host, of small numbers of genes without interfering with other aspects of the recipient's genotype. Thus we could envisage the introduction of genes for the uptake and hydrolysis of sucrose into a production strain that does not grow on sucrose, permitting it to use this sugar instead of glucose.

6. New techniques for genetic manipulation

It is evident that considerable scope exists for the application of genetic techniques in new ways to both the qualitative and the quantitative aspects of antibiotic production. Although members of each of the main groups of antibiotic producing microorganisms have natural systems of genetic recombination, these are rarely ideal for practical purposes, usually because their frequency is either uncharacterized or low compared with that of asexual reproduction in industrially relevant strains (Hopwood & Merrick 1977). Moreover, they are largely confined, by definition, to gene transfers between members of the same species. Fortunately, we are now in an era when rapid advances are being made in *in vivo* and *in vitro* methods of genetic recombination in microorganisms (Hopwood 1979; Queener & Baltz 1979), including the filamentous fungi, actinomycetes and bacilli, which are most important in antibiotic production. The two most relevant techniques are those of protoplast fusion and of recombinant DNA manipulation. Together, they will allow for the two main requirements identified earlier in this article: the ability to bring about efficient generalized recombination between divergent strains or wild-types and the ability to introduce genes from distantly related or unrelated organisms into antibiotic-producing strains.

(*a*) *Protoplast fusion*

Techniques now exist for the fusion of protoplasts from a range of *Streptomyces* species by treatment with polyethylene glycol (Hopwood *et al.* 1977; Baltz 1978; Hopwood & Wright 1978, 1979). Intraspecific protoplast fusion has been reported in two bacilli (Fodor & Alföldi 1976; Schaeffer *et al.* 1976). Protoplast fusion within and between fungal species is also readily achieved (Ferenczy *et al.* 1976; Peberdy 1979).

In the prokaryotic streptomycetes, fusion of protoplasts results, with almost unit probability, in recombination between the naked genomes of the fusing protoplasts (Hopwood & Wright 1978). Moreover, these genomes are, initially at least, essentially complete, in contrast to the situation in the merozygotes resulting from plasmid-mediated conjugation between hyphae (Hopwood *et al.* 1973). Thus, protoplast fusion in *S. coelicolor* A3(2) results in very high proportions of haploid recombinants (up to at least 20% of the unselected output of a fusion experiment) involving all regions of the genome, with many multiple crossover classes, as well as a significant proportion of recombinants involving three or four parental genomes (Hopwood & Wright 1978); these are ideal characteristics for the hybridization of divergent lines in a yield improvement programme. The proportions of recombinants, and of multiple crossover classes among them, can be increased even further by ultraviolet irradiation of the parental protoplasts immediately before fusion, presumably by introducing lethal hits into them, which can be recombined out after fusion (Hopwood & Wright 1979). An intense ultraviolet irradiation of

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one parent provides a way of polarizing the cross, to make one parent a recipient of short chromosomal segments from the irradiated parent (D. A. Hopwood & H. M. Wright, unpublished results).

The subject of interspecific protoplast fusion in streptomycetes has received much less attention. The low frequencies of recombinants so far achieved (Hopwood *et al.* 1978; Godfrey *et al.* 1978) may reflect poor genome homology, the operation of restriction–modification systems, or, conceivably, a 'physiological incompatibility' between the cytoplasms of two strains, each being dedicated, at the moment of fusion, to the synthesis of diverse antibiotics, to which the other may be sensitive. These factors are doubtless capable of being attacked. In particular, if restriction is a problem, restrictionless mutants should be isolable by screening for phage sensitivity (Chater & Wilde 1976). Thus the potential of interspecific fusion to generate new antibiotic structures, as well as to enlarge the pool of yield-enhancing genes, may be realized.

In *Bacillus subtilis* and *Bacillus megaterium*, protoplast fusion also gives rise to frequent generalized haploid recombinants, although not at such a high frequency as in streptomycetes (Foder & Alföldi 1976; Schaeffer *et al.* 1976). Heat inactivation of one parent provided a means of polarizing the crosses (Fodor *et al.* 1978).

In filamentous fungi, protoplast fusion generates intraspecific or interspecific heterokaryons with ease, but the barriers of nuclear fusion and chromosomal segregation must be overcome for true recombinants to arise (Kevei & Peberdy 1979). A breakthrough may have been achieved by Hamlyn & Ball (1979), who fused protoplasts of divergent strains of *Acremonium chrysogenum* and found, instead of heterokaryons or stable diploids, a variety of haploid recombinants that could be isolated directly from the regenerated culture and screened for desirable genotypes.

(*b*) *Recombinant* DNA *techniques*

Several directions might be followed in attempts to exploit *in vitro* recombination approaches in antibiotic production. One would be to clone antibiotic production genes into *E. coli*, thus taking advantage of the wealth of genetic and biochemical information available for this organism. However, we foresee very considerable difficulties in such an undertaking. For example: basic knowledge of the organization of genes for antibiotic synthesis is totally inadequate; large numbers of genes are involved in most pathways, perhaps controlled by several promoters which may themselves be unexpressed in a foreign cytoplasm; the new host may be poisoned by the end product; export of the end product may be inefficient; and removal of unicellular bacteria from the fermentation broth may be comparatively expensive. Thus, although cloning antibiotic production genes into *E. coli* might have considerable analytical value (e.g. for DNA sequencing), we anticipate that the major benefits of recombinant DNA technology to antibiotic synthesis will accrue from the use of host–vector systems endogenous to the antibiotic-producing groups of microbes.

The basic requirements for the application of *in vitro* recombination approaches to these organisms, the availability of vectors and DNA uptake systems, are on the verge of being met. Protoplasts are again crucial to the operation.

Streptomycete protoplasts, treated with polyethylene glycol, will take up plasmid DNA very efficiently, more than 20% of the regenerating population becoming transformed by at least one plasmid molecule at high plasmid:protoplast ratios (Bibb *et al.* 1978), while efficiencies of transfection by actinophage DNA of about 10^{-5} per regenerated protoplast have so far been

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achieved (J. E. Suarez & K. F. Chater, in preparation). For plasmids, removal of natural supercoiling by cleavage and religation reduces the transformation efficiency only slightly (M. J. Bibb & C. J. Thompson, personal communication). Very similar results have been obtained in *Bacillus subtilis* (Chang & Cohen 1979).

Doubtless, the ideal vectors for introducing foreign DNA into streptomyces will themselves be the products of genetic engineering, as in *E. coli*, but early experiments will be possible with available vectors such as the SCP2* plasmid of *S. coelicolor* A3(2) (Bibb *et al.* 1977; Schrempf & Goebel 1977), the SLP1 family of plasmids of *S. lividans* (Hopwood *et al.* 1979; M. J. Bibb, J. M. Ward & D. A. Hopwood, in preparation), and actinophages such as R4 (Chater & Carter 1979). As far as plasmids are concerned, the SLP1 plasmids are attractive because of their fairly small size (relative molecular mass, 6×10^6–8×10^6) and because any sites within the variable segment of (probably chromosomal) DNA possessed by some but not all members of the family are certainly available for cloning without inactivating genes essential for plasmid replication and maintenance. R4 is a promising vector because it is a temperate actinophage with a wide host range, capable of yielding viable deletion mutants (thereby providing space in the virion for foreign DNA segments) and with some suitably located restriction enzyme sites in its DNA (K. F. Chater, unpublished results). Vectors for *Bacillus* species are also being developed (Young & Wilson 1978).

The relative advantages and disadvantages of plasmid and phage vectors for the manipulation of antibiotic genes will only become apparent with experience. It seems probable that most, if not all, applications will require continued expression of foreign genes, so that phage vectors are likely to be useful in the prophage rather than the lytic mode. The low copy number of known streptomycete plasmids and of prophages is likely not to be a disadvantage in those applications in which new combinations of biochemical functions are brought together in the same cell (e.g. in making hybrid antibiotics), though it obviously limits the extent to which increased gene dosage as a means of increasing production will be attainable with these vectors. (But note that the use of highly efficient promoters in low copy number vectors might achieve the same result as increased gene dosage (see §5*d*).)

No plasmid or virus vectors have yet been described for filamentous fungi, but the advances currently taking place following the discovery that plasmid DNA can be transformed into yeast protoplasts treated with polyethylene glycol (Hinnen *et al.* 1978, 1979; Beggs 1978) suggest that recombinant DNA techniques could be developed for organisms such as *Penicillium chrysogenum* or *Acremonium chrysogenum*. Of particular interest is the finding (Struhl *et al.* 1979) that certain segments of yeast DNA, probably containing one of the several origins of replication characteristic of eukaryotic chromosomes, can be cleaved from the chromosomes and ligated to give potential vectors capable of autonomous replication when transformed back into yeast. Perhaps this explains the cytoplasmic inheritance of a transformed phenotype in *Neurospora crassa* (Mishra 1976). It seems probable, therefore, that comparable vectors could now be manufactured to order for any eukaryotic microorganism.

7. Conclusion

The use of genetics in a synthetic approach, the construction of new genotypes by intra- and interstrain recombination, has been emphasized in this article, but the power of genetic manipulation, both natural and artificial, as an analytical tool must not be overlooked. At present,

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nearly all applications of genetics in antibiotic production must have a very large empirical element. In a biometrical approach to yield improvement this will always be inevitable because it will be a superhuman task to understand the operation of the hundreds of genes involved, just as in the development of better yielding strains of plants or animals in traditional, and highly successful, breeding procedures. But, in other applications, such as the generation of new antibiotics, or yield increase by increased copy number or the manipulation of regulator genes, information on the number and location of the genes concerned can be obtained and is, at the moment, limiting. For example, streptomycete plasmids certainly carry genes important for the production of many antibiotics (Hopwood 1978; Chater 1979; Okanishi 1979), but their roles are unclear. Further analysis of these systems, by the genetic techniques now available, can hardly fail to yield information of practical value. For such analytical purposes, as well as for the analysis of the organization and operation of chromosomal genes, cloning on well developed vectors into *E. coli* could well be valuable (see § 6*b*). However, we believe that the imminent availability of endogenous host–vector systems in antibiotic-producing microbes, particularly if they can be complemented by transposon systems of the kind so elegantly exploited in genetic studies of enteric bacteria (Kleckner *et al.* 1977), holds the greatest promise of all for a new horizon in our understanding of the genetic control of antibiotic synthesis.

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Discussion

W. B. Hugo (*Department of Pharmacy, University of Nottingham, Nottingham NG7 2RD, U.K.*). How does PEG 1000 effect protoplast fusion?

D. A. Hopwood, F.R.S. This is not known in detail; most work has been done on eukaryotic systems. Fusion appears to be completely general; for example, yeast protoplasts can be fused to red blood cells.

R. Holliday, F.R.S. (*N.I.M.R., Mill Hill, London NW7 1AA, U.K.*). Why is the recombination frequency so high in protoplasts?

D. A. Hopwood. If the recombination enzymes are synthesized constitutively and there is no physical barrier to recombination between different genomes, high frequencies should be expected.

B. S. Hartley, F.R.S. Why, after millions of pounds have been spent on antibiotic development, is so little known about the genetics of the antibiotic-producing organisms?

D. A. Hopwood. Rigorous structural determination is essential for patenting, while in the past it has been possible to get by without genetics. The research programmes of pharmaceutical companies are therefore largely organized by chemists, not geneticists. Furthermore, university geneticists have sensibly concentrated their efforts on a limited number of organisms.

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