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Invitational ONR Lecture
Genetic Recombination and Strain Improvement

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INTRODUCTION

I am very grateful to the Society for Industrial Microbiology and the Office of Naval Research for this opportunity to talk about some implications of my favorite branch of biology for an important area of applied research. At the outset I confess to a certain bias: I like genetics. It is a good way of looking at biology; it provides a sharp, analytical tool for dissecting complex phenomena; and it is excellent for predicting ways to bring about biological change. I hope to show that, as industrial microbiologists, we cannot afford to go on ignoring the contribution that genetics, suitably combined with other techniques, can make to strain improvement.

DISCUSSION

The Need for Strain Improvement

Very few of the plants and animals used in agriculture in advanced civilizations resemble those found in the wild. The attributes of interest to people differ from those that enable an organism successfully to occupy a natural ecological niche. Thus organisms, the end products of long periods of natural evolution, have been taken and subjected to a rapid process of mutation, crossing, and artificial selection to produce varieties very different from anything that has gone before, and from anything that would occur in Nature in the future. Selection has often been for a single character, such as the yield of grain, wool, or breast muscle, to produce a kind of "monster" that can survive only when cultured under highly artificial conditions (the breasts of some commercial varieties of turkeys are so large that the birds cannot mate; they have to be artificially inseminated).

Exactly the same principles apply to industrial microorganisms. A few commercial processes use strains, or even mixed populations of microbes, that are very similar to those occurring in the wild; but these are a small minority found predominantly in the less artificial microbiological conditions of the food and drink industry. In the frantic environment of large fermentors making antibiotics, amino acids, or enzymes, wild strains are out of place; genotypes able to produce comparatively huge yields of a single product are needed, just as in many branches of agriculture.

Hardly anyone, then, would challenge the need to change the genotypes of microorganisms to make them better industrial citizens, and the faster and farther the better. Disagreements and misunderstandings arise only over the choice of the most appropriate routes to the desired goals. These are varied and a corresponding variety of strategies is needed to take account of differing complexity in the genetic control of product formation. For example, breeding a microbe to convert a feedstock more efficiently into nutritionally high grade biomass requires changes in many genes; maximizing the yield of a single metabolite, such as an antibiotic,

when it is the end product of a long regulated pathway of biosynthetic steps, also needs a surprisingly large number of genetic changes; making more of a single gene product, such as an enzyme, might be achieved by a simple increase in gene dosage; while pursuading a micro-organism to make insulin depends on a qualitative change that would never occur in Nature, and therefore requires a new kind of genetic recombination in the laboratory. In spite of the variety of the strategies, I think that genetic recombination has a contribution to make to all of them. Ironically, examples in the last category, which are technically more challenging and demand more brilliant fundamental research to provide the necessary techniques, may do more to gain general acceptance for genetic recombination in strain improvement, including its less spectacular applications, than has been achieved by two decades of cajoling by geneticists (Pontecorvo 1976) simply because no one can conceive of solving such problems except by the use of recombination.

Recombination in Nature and in Industry

Recombination is the bringing together of pre-existing genetic information into new combinations; parts of two existing genotypes (or part of one and the whole of another) combine to give a new genotype. Recombination operates at all levels of genetic organization, reassorting nucleotide pairs between genes, genes between chromosomes, chromosomes between nuclei, and nuclei, plasmids, or organelles between cells. It is the consequence of several different mechanisms, some known for many decades (crossing over and the reassortment of whole chromosomes at meiosis), others of recent discovery (the operation of insertion sequences and translocating genetic elements), and still others (referred to as "genetic engineering") that make use of natural processes in artificial ways.

What can recombination do that mutation alone cannot achieve? Nothing, qualitatively; it is a question of probabilities. As an extreme example, it would be possible theoretically to derive by mutation a strain of *Escherichia coli* that synthesized insulin. By accumulating successive mutations, and a deletion or two, in a gene coding for a dispensible small protein, a code for the sequence of 80 or so amino acids of proinsulin could be arrived at; it would need considerably fewer than 240 mutational changes, since many nucleotide pairs in the original gene could be conserved, especially in the third codon positions. Yet in practice the problem is well nigh impossible; the probability of all the mutations occurring simultaneously is essentially zero, and their successive accumulation fails for lack of a selection method to recognize intermediate steps in the succession. Hence, people are trying to find a recombinational solution to the problem, bringing together pre-existing genes of mammals and bacteria.

Both the desired genotype and the biochemistry are known in this example, but in many they are not. This does not preclude the use of recombination. On the contrary, plant and animal breeding are full of examples of highly successful recombination programs where selection was for complex characters (such as the grain or breast muscle mentioned earlier), controlled by large and indeterminate numbers of genes whose biochemical roles are likely to remain unknown; often they involve morphogenetic processes about which we are still very much in the dark. The point here is that recombination is an enormously efficient process for generating genetic variation, to be sifted by a suitable selection procedure. We cannot avoid selection in strain improvement, as in evolution.

There is a complete analogy between strain improvement and natural evolution (Hopwood 1974a). In both, a pool of genetic variability is generated from which particular genotypes are selected as parents for the next generation. In the artificial situation, the breeder decides the characters to select, whereas in Nature they are determined automatically by a feedback loop

through reproductive success; those genotypes survive which have an increased probability of reproducing. Assuming a monophyletic origin of life, there could be no genetic diversity without mutation. However, it is very significant that, in virtually every biological group, a system leading to genetic recombination also exists, alongside mutation, and so must surely be adaptive. Recombination is, of course, the *raison d'être* of sexual reproduction, which is present in all groups of eukaryotes, even though certain members of most groups have abandoned it as a short-term expedient, with the consequence of *limiting* variability and so remaining adapted to a fixed environment; but this is an evolutionary dead end. In bacteria the three modes of genetic exchange – transformation, transduction, and conjugation – are distributed widely and they have only the end result of recombination in common; this, rather than the means, is presumably the biologically important character. As for viruses, all those with DNA probably undergo recombination, catalyzed by enzymes that are often coded by their own genes; and they would not carry redundant genes. In contrast, enzyme systems capable of recombining parts of RNA viral genomes are rare or nonexistent. Therefore it is probably no coincidence that many RNA viruses have a genome divided between several RNA molecules, a situation that might appear inefficient in terms of packaging the genes for the next generation, but which has the presumably compensating advantage that it allows recombination (Jaspars 1974). If recombination, alongside mutation, is fundamental to efficient natural evolution, the same is true of artificial evolution.

In this lecture I shall amplify, with some examples, the notion that genetic recombination has an important and largely unexploited role to play in the production of new industrial microorganisms. The examples are largely prokaryotic, but this does not mean that eukaryotic microbes are not amenable to genetics. It is simply that my own experience is with prokaryotes, and they do have some attractive genetical features.

An Empirical Use of Recombination

Recombination need not be used empirically but it can be. We need not necessarily know how many genes control a character, what their roles are, or how they are linked. For most quantitative characters this is always going to be the case. This example concerns breeding for increased antibiotic production by an actinomycete. I have heard of its attempted use in two or three industrial companies and apparently, as expected, it worked; too bad that those concerned are not free to tell us the details. Although I shall talk about antibiotics and about actinomycetes, a similar approach could be used for other products and in any bacterium with a generalized recombination system. Conjugation is the most appropriate because it leads easily to the generation of recombinants in respect of long segments of the genome. Outside the actinomycetes, conjugation is found in the Enterobacteriaceae, which has a few industrially important members, and in the genus *Pseudomonas* (Holloway 1975), which has several. Recent ways of introducing plasmid-mediated conjugation into bacteria which do not already have it could be very useful (Beringer and Hopwood 1976; de Graaff et al. 1973; Haas and Holloway 1976; Towner and Vivian 1976).

Recombination through conjugation in actinomycetes. Fortunately, conjugation is widespread in the important antibiotic-producing genera of actinomycetes (Hopwood 1976) – *Streptomyces, Micromonospora, Nocardia,* and their relatives – which account for about two-thirds of the known antibiotics (Bérdy 1974). The genetic determination of conjugation in these bacteria so far has been studied only in *Streptomyces coelicolor*, and it turns out that plasmid sex factors are involved (Hopwood et al. 1973). Their interaction with the chromo-

some determines different roles in conjugation – recipient, low frequency donor, high frequency donor – much as in *E. coli*. Probably the situation will be similar in other actinomycetes, but in the empirical approach to strain improvement, we can ignore fertility differences and make use of the recombination that usually occurs, at a low but useful level (10^{-5} – 10^{-6}), between strains that have recently diverged from a common progenitor. In a cross between two such strains (experimentally this is just a mixed culture on agar), mating occurs between a few individuals to produce a heterogeneous collection of zygotes. Each contains a complete circular chromosome from either of the parents and a more or less random fragment (on average about a fifth) of the chromosome of the other. Crossing-over between circle and fragment generates recombinant circles that carry reassortments of any genetic differences that distinguished the parents; the problem is that recombinants are outnumbered one hundred thousand to a millionfold by the two parental genotypes produced asexually. A selection for recombinant spores must therefore be made, not at this stage for improved genotypes, but simply for recombinants in general. This would not be necessary if we could switch the organism from asexual to sexual reproduction, but it is one of the features of bacteria that the two processes occur together, although "ultra-fertile" crosses in *S. coelicolor* come very close to giving us wholly recombinant populations.

The required selection is achieved by introducing counterselectable "marker" mutations into each parent before making the cross and selecting recombinants in respect of these markers. By far the most suitable markers are nutritional; a different auxotrophic mutant of each parent is isolated and prototrophic recombinants are selected. Any deleterious side effects of the marker mutations are then irrelevant because members of the selected recombinant population do not inherit them; and the process can be repeated indefinitely for further crosses. If antibiotic resistance markers were used, the mutant instead of the wild-type alleles would be selected and the recombinants would suffer any disadvantages conferred by the markers. Moreover, one would rapidly run out of suitable resistances to use as markers for subsequent crosses.

Auxotrophs can be isolated reasonably efficiently in many streptomycetes, particularly after intense mutagenesis, but some strains yield very few good auxotrophs. What we need is for somebody to develop a really efficient procedure for isolating auxotrophs, similar to the penicillin or cycloserine enrichments for eubacteria. Not only would this allow the rapid isolation of auxotrophs in "difficult" strains but the ability to select spontaneous mutations would avoid the possible induction of unwanted extra mutations along with auxotrophy.

The recombinants obtained with a particular pair of selective markers are not a random sample of all recombinants; they exclude regions of each parental chromosome around the counter-selected marker. Use of alternative pairs of selection points, distributed systematically around the chromosome, ensures a more random sample. But how can we find the time to build a linkage map of the organism and map every mutation before using it in the breeding program? This problem is largely illusory. Mapping studies need not be very time-consuming: in both *Streptomyces glaucescens* (Baumann et al. 1974) and *Nocardia mediterranei* (Schupp et al. 1975) one person working for less than 3 yr was able to build a map of 15-20 loci from scratch, starting with a quick mapping procedure (Hopwood 1974b) ideally suited to this group of bacteria. But more than this, most of the genes recognized by auxotrophic mutations, which control pathways of biosynthesis of primary metabolites, may be arranged in a conserved pattern throughout the genus *Streptomyces* and into the section of *Nocardia* represented by *N. mediterranei*. It may go wider than this; there is no linkage map yet for *Micromonospora* or the other genera of actinomycetes now being screened for new anti-

biotics. This illustrates the conservation of gene arrangements in bacteria over comparatively long periods of evolution, and it could work in our favor. Even in *S. coelicolor* the basic biochemical genetics has been largely ignored so that we know the precise phenotype of the mutants, and therefore the functions, of only a small minority of the mapped genes. We need a cooperative effort to fill some of the gaps and so identify a minimum of about half a dozen well-spaced markers, with unique phenotypes easily recognized by simple nutritional tests. The approximate map positions of equivalent auxotrophs in a strain being subjected to recombinational improvement could then be assumed with reasonable certainty, obviating the need for mapping. Selection of recombinants between a number of pairs of markers would ensure a satisfactorily random sample of recombinant progeny for the next stage of the program.

The next stage is a screening of the recombinants for those with increased yield. In the mutation plus recombination plus selection program that I am proposing, this screening of recombinants is no different from that of the survivors of mutagenesis in a conventional mutation plus selection program and therefore I do not need to discuss it; industrial microbiologists have spent a great deal of effort on working out screening techniques.

The case for recombination. This, then, is the procedure. Why and how should we use it? Just as in natural evolution, we have to strike a balance between mutation and recombination. In a mutation plus selection program, a single straight lineage connects the current best production strain all the way back to the wild-type organism from which it is descended. After each round of mutagenesis, the best performing survivor probably was chosen for the next round. A lineage of 10 rounds includes only 10 new genotypes, although more than 10 allelic substitutions may well be involved since many of the rounds probably introduced more than one mutation into the chosen survivor. A backcross of the current production strain to the wild-type could therefore generate more than 1024 (2^{10}) different genotypes – more, by a factor of 2, for each "extra" mutation in any round of mutagenesis – of which only 10 definitely have occurred before. On statistical grounds the genotype at the top of the selected lineage is unlikely to be the highest yielding of the 1024 possible genotypes, or the best starting point for further mutations, provided that two conditions are true. The number of potentially mutable genes that can affect yield must be large compared with the number of rounds in a typical mutation plus selection program; and many alleles interact nonadditively, the effect on yield of one mutation depending on which other mutations are present in the genotype. The truth of the first condition follows from the results of all quantitative genetic studies of other systems (Caten and Jinks 1976); from the fact that yield improvement by mutation always occurs in a long succession of steps; and from the results of the one biometrical study of antibiotic yield that has been made (Merrick 1976). It means that optimizing yield is not a question of generating a unique genotype involving a small number of genes, and that those mutations that have been selected in the lineage are only a small fraction of those that might have been selected. The significance of the second condition has to be assessed more indirectly. Nonadditive genetic variance is a fact of life, but to be entirely convincing it will have to be demonstrated for yield genes in a reasonably high yielding antibiotic producer. In Merrick's (1976) study, involving natural variation in *Aspergillus nidulans*, most of the variance was additive, but more was nonadditive when some induced variation was analyzed (I. Simpson and C. E. Caten, pers. comm.) and even then high yields had not been reached. I would predict that nonadditive interactions will become more and more significant at higher and higher yields and under more intense mutagenesis.

The consequences of the situation are that the possibilities for mutating to higher yield at

any particular stage in a lineage are limited by what has, or has not, gone before. A mutation that was good a couple of rounds ago may be a liability preventing a further series of potentially useful changes from being recognized. Or, starting from a particular genotype, a really significant yield increase might need two mutations neither of which alone would have a recognizable effect. We see the value, therefore, of a judicious use of recombination to reassort the mutations. Since genetic variation drops to zero whenever the pedigree of a haploid organism passes through a single individual, genetic variability for future release can be retained only by a suitable use of multiple selection lines, with intercrosses and backcrosses from time to time to generate further selection lines. The best network of mutation and recombination needs to be found, and some further biometrical studies are needed to evaluate possible schemes. In the meantime we shall have to rely largely on trial and error.

A commonly heard counterargument, that mutation plus selection has been "fantastically successful" in increasing antibiotic yields, is specious. Certainly yields have been improved very greatly, but it does not follow that the selection programs have been efficient in materials, time, and effort – and these have been expended on a tremendous scale – because there has been no serious comparison with a combined mutation and recombination approach.

Interstrain crossing. An unknown factor is the extent to which crosses between lines derived from different wild-types will be possible. They would greatly increase the gene pool available for recombination, just as in plant breeding where wild grasses or native Andean solanums have been invaluable sources of desirable genes for introduction into cereal or potato stocks, to mention just two examples. For the predictable transfer of small numbers of genes, plasmid technology, both in vivo and in vitro, will be very useful as we see in subsequent examples. But in the empirical approach with a polygenic character, recombination of whole genomes is required. Three factors might limit random interstrain recombination: restriction systems; the possible specificity of the mating reaction; and imperfect genome homology. They will need to be assessed and, if necessary, overcome. The key is restriction, since only when it is absent or quantified can we assess the relevance of the other factors.

Restriction in streptomycetes only now is being demonstrated for the first time (Chater and Wilde 1976). In a first screen for restriction, one may use in vitro enzymological or in vivo bacteriophage tests. However, restriction may still be missed in vitro because target DNA's happen to lack the appropriate sites, and in vivo because a strain may restrict a test phage so efficiently that it fails completely to plaque on that strain, which therefore appears to lie outside its host range (K. F. Chater, pers. comm.) or simply because phages may not have sites for enzymes present in their normal host. Isolation of nonrestricting mutants by virtue of increased phage sensitivity (Chater and Wilde 1976) may reveal such systems and, of course, also eliminates them.

The mating reaction in *Streptomyces* shows apparent specificity: transfer of the SCP1 plasmid from *S. coelicolor* A3(2) to *Streptomyces lividans* 66 or *Streptomyces parvulus* ATCC 12434 was less frequent than transfer within each strain, though not by a large factor, whereas transfer back to *S. coelicolor* A3(2) was much less frequent; transfer between *S. coelicolor* and *S. griseus* CUB94 or *S. purpurochromogenes* CUB515 was barely detectable in both directions, but again was very efficient in the new host (Hopwood and Wright 1973; and unpublished results). In the case of the *S. coelicolor/S. lividans* pair, no evidence was found that restriction was responsible, but in the light of the considerations discussed above, this must remain a possibility. Further studies must be made in order to distinguish any real strain specificity of the mating reaction from restriction effects. If strain specificity is severe, it may

be possible to overcome it by protoplast fusion, an exciting possibility for facilitating wide genetic exchanges (Fodor and Alföldi 1976; Schaeffer et al. 1976).

Genome inhomology can be a real cause of poor interstrain fertility in bacteria, but its importance may have been overestimated (Hopwood 1973). I would predict that, when crosses become possible between strains known to be nonrestricting, and where plasmid transfer can be used to monitor mating, genome inhomology will be found not to limit interstrain recombination too severely. Even between two bacteria as comparatively divergent as *E. coli* and *Salmonella typhimurium*, inhomology is severe only over certain regions of the chromosome (Middleton and Mojica-a 1971).

The need for more knowledge. I hope that I have made a convincing case for recombination in an empirical strain improvement program. If it is accepted, there is a clear need for some further studies in streptomycete genetics: development of a selective auxotroph isolation procedure; some biochemical genetics of auxotrophs; a systematic study of restriction and interstrain recombination; and a real biometrical comparison of various forms of breeding scheme to gain practical insight into the parameters involved.

The Informed Use of Recombination

Since most quantitative characters depend on many genes playing diverse biochemical roles, we are unlikely to want to spend the considerable effort needed to identify them sufficiently accurately for recombination in completely predictable ways; hence the empirical approach just discussed. The deliberate construction of useful genotypes is more immediately feasible when qualitative changes can result from the recombination of comparatively small numbers of identifiable genes or when significant quantitative changes result from the action of mutations in regulator genes or promoters as in the case of amino acid production (Nakayama et al. 1976). There may be scope for recombination to bring sets of genes under the influence of more efficient promoters or to free them from the control of regulators.

Recombination in an analytical role. Recombination plays an analytical as well as a constructional role in such breeding programs because gene identification itself requires recombinational analysis. However, knowledge of the genetics of the production of useful products by industrial microorganisms is extremely slight and this severely limits the use of genetics in many aspects of strain improvement until the situation is remedied. Again taking antibiotic synthesis as an example, it is almost unbelievable how little we know about the location of the structural genes involved, not to mention possible regulator genes. Three groups of microbes produce medically important antibiotics: filamentous fungi of the genera *Penicillium* and *Cephalosporium*; eubacteria of the genera *Bacillus* and *Pseudomonas*; and actinomycetes of the genera *Streptomyces, Nocardia,* and *Micromonospora*. For no commercially important member, except *Streptomyces rimosus*, has a significant proportion of the genes been identified, and in most cases none of them have. A sufficient reason is usually the lack of the recombination studies needed to generate a linkage map against which to observe the segregation of mutations abolishing or modifying antibiotic production. For the industrial fungi, the parasexual cycle has been surprisingly difficult to develop into a useful mapping situation (Ball 1973; Calam et al. 1973; Nüesch et al. 1973). There appear to be no published results of attempts to study recombination in commercially important bacilli or pseudomonads. In the actinomycetes significant recombination analysis has been carried out on only two streptomycetes making important antibiotics (chloramphenicol and oxytetracycline) and on

Nocardia mediterranei, the producer of rifamycin, while evidence that recombination is available in strains producing four other major antibiotics (chlortetracycline, erythromycin, neomycin, and streptomycin) has not been extended to a useful analytical level (Hopwood 1976).

It is encouraging that, in each of the three major groups of microbes, there is now available an "academic" organism with a well-developed genetic system which makes one or more antibiotics and which therefore could be an informative model for the study of antibiotic genes. Such investigations could provide information and general concepts that would make subsequent attempts to identify and manipulate antibiotic genes in commercial strains quicker and more efficient. The organisms are: *Aspergillus nidulans*, a producer of penicillin; *Bacillus subtilis* which makes several antibiotics of the class to which gramicidin, tyrothricin, and bacitracin belong; and *Streptomyces coelicolor* which makes actinorhodin and methylenomycin. Identification of penicillin genes in *A. nidulans* has begun (Holt et al. 1976) and progress has been made with genes for the two antibiotics in *S. coelicolor* (Wright and Hopwood, 1976a,b), but the antibiotics of the genetically studied strains of *B. subtilis* largely have been disregarded except insofar as pleiotropic sporulation mutations tend to abolish antibiotic production (Schaeffer 1969).

A genetic study of penicillin mutants in *A. nidulans* will undoubtedly illuminate the biosynthetic pathway but probably, like nearly all metabolically related sets of genes in eukaryotes, they will be found scattered over the chromosomes. The homologous genes will need to be identified afresh in *Penicillium chrysogenum* for manipulation purposes, but the availability of a stock of characterized mutants in *A. nidulans* will greatly simplify the task. In prokaryotes, in contrast, functionally related genes tend to be arranged in clusters to varying degrees. Significant clustering of antibiotic genes undoubtedly will be found, at least in streptomycetes; those for oxytetracycline in *S. rimosus* (Boronin and Mindlin 1971) and for actinorhodin in *S. coelicolor* (Wright and Hopwood 1976b) are strongly clustered on the chromosome. Clustering of primary metabolic genes in bacteria tends to have regulatory significance and so it is not impossible that the same is true of secondary metabolic pathways and that important regulatory genes may be recognized by the effects of mutations on the expression of clustered genes, offering an exciting challenge for future work.

In bacterial genetics a special significance attaches to the arrangement of genes on plasmids, itself an aspect of gene clustering, and here too there will be important findings to be made in relation to antibiotic synthesis. It seems certain that the structural genes controlling the unique steps in methylenomycin synthesis in *S. coelicolor* are linked on the SPC1 plasmid (Kirby and Hopwood 1976) and there is enough evidence of plasmid involvement in the synthesis of other antibiotics, notably chloramphenicol (Akagawa et al. 1975) and turimycin (Kähler and Noack 1974), to suggest that many other examples must exist.

Biochemical genetics of antibiotics, a worthy challenge. The conclusion I draw from these considerations is that the time is over-ripe for an intensive study of the biochemical genetics of antibiotic pathways (and this is only an example; the same is true of other useful products). Apart from its practical implications it is a worthy topic for academic investigation since gene-enzyme relationships in many antibiotic pathways are unlikely to have exact counterparts in the well-studied primary metabolic pathways of *E. coli* and *S. typhimurium*. Some good examples may be: the assembly of the tripeptide skeleton of the β-lactams; the non-ribosomal oligopeptide synthesis exemplified by the antibiotics of bacilli; and the involvement of multi-enzyme complexes in the condensation of acetate or propionate units for the synthesis of many actinomycete antibiotics. In some of these cases, extensive biochemical studies

have already been made (Corcoran 1976; Fawcett et al. 1976; Frøyshov 1975; Lancini and White 1976; Lipmann, 1973; Vaněk et al. 1971) and it could be a question of initiating or extending the genetics of the producing organisms to complement them.

Plasmids and Strain Improvement

Plasmids (and bacteriophage genomes may be considered as a special case) have unique significance in strain improvement for two reasons both of which arise from their ability to exist and replicate separately from the bacterial chromosome. They allow foreign genes that have no counterpart in an organism and therefore could not be introduced into its genome by recombinational *replacement* (crossing over) to be *added* to its genotype, and they allow the selective amplification of certain genes of an organism (or of a different organism), and therefore of their products. The latter in principle could result from the reduplication of chromosomal genes, and there are examples of such tandem repeats associated with increased product formation (Collins 1976; Hartley 1974), but only for genes of the same organism and in a less dramatic and predictable way than by the use of plasmids.

Plasmids in the strict sense have not been found in eukaryotes, but other genomes undoubtedly will be adapted to some of the same ends. DNA-containing animal viruses will probably be used as vectors of foreign DNA in animal cell cultures; viruses of the cauliflower mosaic type might be used for plant cells; minicircular DNA (Guerineau et al. 1974) could be a possible candidate in yeast; and when methods for splicing RNA molecules are developed, the genomes of fungal viruses (Lemke et al. 1976) could be useful. However, I shall discuss only examples where the genotypes of prokaryotes are modified by plasmid manipulation.

There are three classes of plasmid experiments with industrial relevance: situations in which the natural occurrences of useful genes on plasmids can be exploited, such as hydrocarbon degradation in pseudomonads and antibiotic production in streptomycetes; those in which in vivo phenomena are harnessed to transfer useful genes to plasmids; and in vitro plasmid manipulations.

Useful natural plasmids. Certain strains of *Pseudomonas putida* and *P. oleovorans* harbor plasmids controlling the degradation of a variety of hydrocarbons (Chakrabarty 1976; Wheelis 1975). Many of these plasmids (for camphor, salicylate, toluate, or naphthalene) are sex factors able to promote their own transfer from cell to cell by conjugation, while others (for octane and xylene) require a separate sex factor to promote their transfer. Most of the plasmids belong to different compatibility groups and so can be brought together in a recombinant strain with the combined catabolic capabilities of the separate strains from which the plasmids were derived (Friello et al. 1976). This is an excellent example of the manipulation of naturally occurring plasmids in strain construction.

While identification of the *Pseudomonas* plasmids depended initially on recombination studies – transfer of the relevant phenotype to recipients at a much higher frequency than chromosomal markers – circular DNA corresponding to most of them has now been isolated (Chakrabarty 1976). This is not true of *Streptomyces* antibiotic plasmids, which have so far been recognized by genetic criteria only. However, evidence for the existence of at least some of them is strong and others will surely be discovered. How could they be useful? In contrast to the *Pseudomonas* case, a strain harboring plasmids for the synthesis of several different antibiotics might not be an advantage industrially. However, a strain carrying two plasmids (or one plasmid and a set of chromosomal genes) determining two related antibiotic pathways could be advantageous if it produced a "hybrid" antibiotic. This may not be too unlikely

because of an interesting property of many enzymes catalyzing steps in the biosynthesis of secondary metabolites, their relative "lack of specificity" in the following sense. The later steps in the biosynthesis of secondary metabolites, after a basic structure such as a ring system has been synthesized, are the addition of functional groups at various points which determine, for an antibiotic, the antimicrobial properties of the molecule or distinguish it from other members of the same antibiotic family. An enzyme can often operate irrespective of whether another enzyme has done its job. Thus in the wild-type a metabolic "grid" rather than a linear pathway may lead to an end product (Bu'lock 1971), while mutational loss of a particular enzyme, even in a pathway that is essentially linear in the wild-type, can fail to interrupt the pathway, which is carried on by the later enzymes to give an abnormal end product lacking a particular functional group. A good example concerns the tetracycline family, where a binary hierarchy of compounds results from loss of members of a series of pathway enzymes (Vančk et al. 1971). A practical exploitation of this catholic taste of the enzymes of antibiotic pathways is the production of new antibiotics by feeding artificial precursors with unnatural combinations of functional groups to mutant strains, the technique of "mutational biosynthesis" (Nagaoka and Demain 1975). A use for plasmid transfers will come when parts of two natural pathways can be put together in one strain, obviating the necessity to synthesize and feed precursors.

Another application of natural plasmids could be the transfer of antibiotic production to a strain with more desirable fermentation properties than the original strain, for example, because its growth habit was more suitable or because it grew on cheaper substrates. Early in an improvement program for a new antibiotic, its synthesis could be transferred to a series of other strains, of a variety of species (the SCP1 plasmid of *S. coelicolor* causes methylenomycin production when transferred to *S. lividans* or *S. parvulus*), which could be evaluated as potential candidates for yield improvement. This could proceed by methods essentially the same as for a chromosomally determined antibiotic since most yield-enhancing mutations will be chromosomal even when the pathway genes for antibiotic synthesis are plasmid borne. In contrast to the concn of the gene products themselves (Brammar 1976; Collins 1976), flux through a multi-enzyme pathway is not likely to be significantly "limited" by dosage of the pathway genes at the start of an improvement program (Kacser and Burns 1973), so that direct selection for mutations increasing plasmid copy number would not result in a significant yield increment, but the beauty of a breeding program in a plasmid-bearing strain is that such mutations could occur and be recognized at any stage in the program at which an increase in gene dosage became relevant.

The construction of new plasmids. Any uses for plasmids naturally carrying desirable genes apply equally to situations in which genes are transferred to a plasmid from some other source – the chromosome of the same or a different strain, or a second plasmid – by in vivo or in vitro techniques. In vivo recombination of nonhomologous genes has been known for many years: examples are F′ formation in *E. coli*; the formation of specialized transducing particles of λ and φ80 *E. coli* phages; and the association of segments of R factors to generate new combinations of antibiotic resistances. The processes underlying at least some of these "illegitimate" recombinations are now becoming much clearer with the study of insertion sequences, transposable genetic elements and bacteriophage Mu (Cohen 1976; Howe and Bode 1975; Starlinger and Saedler 1976). In making use of such phenomena in strain construction, a suitable selection for the desired genotype is fundamental, but knowledge of the special properties of transposable elements and of Mu certainly helps in making rational choices of

suitable crosses and selections. However, as is usually the case, the knowledge hardly extends outside *E. coli* and *S. typhimurium*. Although, given time and effort, the necessary in vivo technology could undoubtedly be developed for industrial prokaryotes, it may well be that resources will be better spent on the development of suitable systems for in vitro plasmid recombination ("genetic engineering") in these organisms since they are likely to be more versatile and predictable for the transfer and amplification of genes from the same or closely related organisms, and at the same time offer the unique advantage of allowing transfers from completely unrelated organisms.

The main thrust of work in genetic engineering is currently the refinement of methods for cloning foreign DNA in *E. coli*: the development of even more suitable plasmid and phage vectors, including their greater amplification and provision with more efficient promoters for the transcription of inserted genes; of more versatile methods of inserting nonhomologous DNA segments into them; and of methods for the recognition of clones carrying desired DNA sequences. In experiments in which the cloned DNA sequence itself is all that is needed, there is probably no good reason for going outside *E. coli*, but for industrial applications in which gene products are required, the potential field is much wider. One class of recombinations will use the host bacterium to transcribe and translate information borne entirely on the introduced DNA segment – the well-worn example of insulin could be a case in point – and a suitable host could perhaps be any safe and productive bacterium; it might or might not be *E. coli*. In fact, we shall surely not be content until the new host produces the desired product in higher and higher yield and the genotype of the host will have an important influence on this. So the choice of host may in fact be much more critical than might at first appear; and there will be endless possibilities for the improvement of the strain – by the now familiar trinity of mutation, recombination, and selection – after the original introduction of foreign genes has been achieved.

Another class of experiments will use a combination of host genes and those on the introduced DNA to yield a product such as the "hybrid" antibiotic discussed above, and here the choice of host is severely circumscribed. Hence there will be a need to develop cloning vectors for each of the industrially important genera and a start has already been made at least in *Pseudomonas* (Chakrabarty 1976) and *Streptomyces* (D. A. Hopwood, M. J. Bibb, and S. N. Cohen, unpublished results). When vectors are available, the problem of selecting clones carrying the desired genes will arise. A random or "shotgun" approach may succeed when the product of a single gene or gene cluster is needed, but for any more complex situation knowledge of gene locations will be invaluable since this could allow the predictable combination of two or more genes or gene clusters on the same cloning vector, perhaps by selection for markers known to be closely linked to the desired genes. This is another example of the practical value of linkage mapping.

Conclusion

I have indicated in this lecture something of the diversity of genetic changes that come under the heading of genetic recombination. I have tried to choose realistic examples, perhaps not always the most imaginative, of possible applications of recombination to strain improvement rather than concentrating on its more futuristic aspects. As I said at the beginning, genetic engineering may help to gain acceptance for recombination in general in this field, provided that it is recognized as a particular example of a recombinational technique. Certainly it is an exciting one, but alone it is not the answer to all our prayers. Its most obvious applications are in the wide transfer of single genes, and its least obvious are in the synthesis of desirable genotypes for quantitative characters controlled by large numbers of scattered genes. In

between is a challenging spectrum of applications where there is almost endless scope for the ingenious blending of in vitro and in vivo recombination techniques and of the knowledge that they yield.

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