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Contribution of genetics to the production and discovery of microbial pharmaceuticals

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Recombinant DNA technology, hybridoma technology, enzyme engineering and protein engineering are all parts of the new biotechnology which is having a major effect on research in health care, diagnostics, agriculture and promises to make inroads in the practices of other industries such as petroleum, mining, foods and chemicals. Genetics has had a long history of contributions to the production of microbial pharmaceuticals, especially antibiotics. The tremendous increases in antibiotic productivity and the resulting decreases in costs have come about mainly by mutation and screening for higher producing microbial strains. Although mutation was used for forty years to improve antibiotic production, genetic recombination had been virtually ignored. Recently, a type of genetic engineering called "protoplast fusion" changed the situation markedly.

Of greatest excitement is the application of recombinant DNA technology to the production of antibiotics and entire antibiotic pathways have already been cloned. The development of recombinant DNA technology is having its major impact on the production of rare polypeptides which are enzymes, hormones, antibodies or other regulatory agents. As a result, mammalian polypeptides can now be classified as "microbial pharmaceuticals." In addition to natural polypeptides, analogs can be produced by recombinant DNA technology and this has added an extra dimension of excitement to the field. The future is thus insured for the expanded use of microorganisms in the pharmaceutical world and the continued improvement in microbial processes to reduce the cost of such drugs.

INTRODUCTION

Although industrial microbiology is not a new field, the recombinant DNA (rDNA) discoveries made some 15 years ago have propelled the field to new heights and have established a new biotechnology industry around the world. Biotechnology has extended itself into all walks of life and today there is hardly a company or an industry that either isn't in biotechnology or wondering why it isn't.

Recombinant DNA technology, hybridoma technology, enzyme engineering and protein engineering are all parts of the new biotechnology which is having a major effect on research in health care, diagnostics, agriculture and promises to make inroads in the practices of other industries such as petroleum, mining, foods and chemicals. The progress in this field has been truly remarkable. Within 4 years after the discovery of rDNA, genetically engineered bacteria were making insulin, somatostatin and growth hormone. This led to an explosion of investment activity in new companies, mainly dedicated to innovation via genetic approaches. Newer companies are now entering the scene in various niches such as bioprocess technology and downstream-processing. Auxiliary industries have benefitted in the commercial world of biotechnology by supplying equipment, supplies, information and mechanisms of communication.

Many companies have invested in biotechnology with no clear idea of the future but with the faith that genetics will lead to products that cannot even be conceived of at the present time. Today there are about 200 small biotechnology companies and about 50 large pharmaceutical, chemical and energy companies in the U.S. alone devoting all or part of their resources to biotechnology. Investments in the field of genetic engineering have amounted to several billion dollars in the past ten years. Some of the biotechnology companies are finally showing profits although these profits are small compared to their total spending on research and development (ca 100 million dollars per year). Most other small companies are spending more than they are bringing in and some of these are in bad financial condition. Some large companies are investing heavily in the construction of major new laboratory complexes devoted to life sciences.

Genetics has had a long history of contribution to the production of microbial pharmaceuticals, especially antibiotics. The tremendous increases in antibiotic productivity and the resulting decreases in costs have come about mainly by mutation and screening for higher producing microbial strains. In the main, this has been "brute force" screening but in recent years, efforts have been devoted to miniaturization and automation of screening procedures and to methods of enhancing the frequency of improved strains by selection procedures, e.g., the isolation of antimetabolite-resistant mutants in cases where the natural metabolite is a precursor, an inhibitor or a corepressor of a biosynthetic pathway. For example, four of bacitracin's twelve amino acid residues are branched amino acids and leucine is known to stimulate bacitracin production. Deregulation of branched amino acid synthesis by selection for resistance to azaleucine (1) led to an improved bacitracin producer (400% in defined medium with a decreased dependence on exogenous leucine; 20% superiority in complex medium). Transaminase B activity in the mutant was found to be 5 to 10 fold derepressed.

Mutation has also served to shift the proportion of metabolites produced in a fermentation broth to a more favorable distribution, to elucidate the pathways of secondary metabolism, and to yield new antibiotics (2). Some examples follow.

Valine (via isobutyrate) is a precursor of the butyrate moiety of monensin A. By selection for resistance to the valine antimetabolites, 2-amino-3-chlorobutyrate and norleucine, Streptomyces cinnamonensis double mutants were obtained with improved production of the desirable component, monensin A (3). The less desirable component, monensin B, does not have a butyrate unit. Whereas the parent produced 50% each of monensins A and B, the double mutant produced 90-93% monensin A and about the same total titer as the parent. Ethionine resistance did not improve the A/B ratio but doubled the total titer, methionine being the donor of the monensin methyl group. The commercially useful antibiotics demethyltetracycline and adriamycin were discovered by simple mutation of the cultures producing tetracycline and daunomycin, respectively. Blocked mutants have also been very helpful in conversion of plant sterols to the steroid ring nucleus. The technique of mutational biosynthesis (4) has been used for the discovery of many new aminoglycoside, macrolide and anthracycline antibiotics.

Although mutation had been used for forty years to improve antibiotic production, genetic recombination had been virtually ignored. The principal reason was the low frequency of recombination, e.g., 10^{-6}. However, a type of genetic engineering called "protoplast fusion" (5) has changed the situation markedly. Today frequencies of recombination have increased to even greater than 10^{-1} in some cases, and strain improvement programs routinely include protoplast fusion between different mutant lines. Successes have been obtained in the cephalosporin and cephamycin strain improvement programs (5).

Another use of protoplast fusion has been the recombination of different strains from the same or different species to yield new antibiotics (6). Such has been the case with anthracyclines, aminoglycosides and rifamycins. Protoplast fusion has also been useful in elimination of an undesirable component from penicillin broths (7).

Of great excitement is the application of recombinant DNA technology to the production of antibiotics (8). The genes encoding individual enzymes of antibiotic biosynthesis which have already been cloned include those of the cephalosporin, clavulanic acid, prodigiosin, undecylprodigiosin, actinomycin, and candicidin pathways (9). The isopenicillin N synthetase ("cyclase") gene of Cephalosporium acremonium has been cloned in Escherichia coli and expressed at a level of 20% of total cell protein (10). Cyclase in Penicillium chrysogenum (11) and Streptomyces clavuligerus (12) have also been cloned. The expandase/hydroxylase gene of C. acremonium has been cloned in E. coli (13). The protein accumulated to a level of 15% of total cell protein as inclusion bodies. In addition, a large number of antibiotic resistance genes from antibiotic-producing organisms have been cloned and expressed.

The potential use of recombinant DNA technology in antibiotic improvement and discovery has been enhanced by the finding that some streptomycete antibiotic biosynthetic pathways are coded by plasmid genes, e.g., methylenomycin A. Even when the antibiotic biosynthetic pathway genes of streptomycetes are chromosomal, they appear to be clustered into operons (14) which facilitate transfer of an entire pathway in a single manipulation. Clustering has been observed with seven structural genes of actinorhodin biosynthesis, five genes of undecylprodigiosin biosynthesis in Streptomyces coelicolor, eight genes of chloramphenicol synthesis in Streptomyces venezuelae and three genes of rifamycin biosynthesis in Norcardia mediterranei. The genes of oxytetracycline (OTC) biosynthesis in Streptomyces rimosus are in two clusters, opposite each other on the map (15). The steps before anhydrotetracycline (ATC) are in one cluster and the genes after ATC and the genes for cosynthetic factor I biosynthesis are in the other cluster. Cosynthetic factor I is involved in the reduction of 5a(11a)-dehydro OTC to OTC. Also present in these actinomycete biosynthetic clusters are regulatory and resistance genes. On the other hand, fungal genes coding for penicillin and cephalosporin biosynthesis are distributed among different chromosomes.

Of great significance has been the cloning of entire antibiotic pathways. The genes of the actinorhodin pathway, normally clustered on the chromosome of S. coelicolor, were transferred en masse via a plasmid to Streptomyces parvulus and were expressed in the latter organism (16). Similarly, the cloning of the entire erythromycin pathway (25-30 enzymes) has been accomplished (17). By cloning DNA from the erythromycin-producing Streptomyces erythreus into a cosmid vector bifunctional for E. coli and Streptomyces, an E. coli clone carrying erythromycin biosynthetic genes was identified by hybridization to a cloned erythromycin resistance gene from S. erythreus. A plasmid was isolated and introduced into Streptomyces lividans. As a result, transformants produced erythromycin A.

Continued progress in the application of recombinant DNA to antibiotic production should lead to overproduction of limiting enzymes of important biosynthetic pathways, thus markedly increasing production of the antibiotic. Of course, these considerations also apply to non-antibiotic secondary metabolites such as enzyme inhibitors, immunomodulating agents, and antiparasitic compounds.

With respect to the discovery of new or modified antibiotics, recombinant DNA techniques can be used to introduce genes coding for antibiotic synthetases into producers of other antibiotics or into non-producing strains to obtain modified or hybrid antibiotics. In recent work, gene transfer from a streptomycete strain producing the isochromanequinone antibiotic actinorhodin into strains producing granaticin, dihydrogranaticin and medero-mycin (which are also isochromanequinones) led to the discovery of new antibiotic derivatives, mederrhodin A and dihydrogranatirhodin (18).

The development of recombinant DNA technology is having its major impact on the production of rare polypeptides which are enzymes, hormones, antibodies or other regulatory agents. As a result, mammalian polypeptides can now be classified as "microbial pharmaceuticals". Rather early in this remarkable development, it was realized tha the same mg quantities of mammalian polypeptides such as somatostatin that were produced in a few liters of recombinant E. coli broth previously had to be extracted from the brain tissue of a half a million sheep. Today mammalian polypeptides are being produced in bacteria and yeast at a level of 1 to 50% of cell protein and concentrations of 10 mg to 5 g per liter. It is clear that the excessive pre-recombinant DNA prices of interferon (and other poly-peptides), their relative unavailability, and lack of purity, are things of the past. Those products which are already approved for use in medicine are human insulin, human growth hormone and alpha interferon. Among those being developed at the present time include other interferons, anti-hemophelia factor (Factor VIII), human tissue plasminogen activator, interleukins and erythropoeitin.

Recombinant DNA methodology yields purer proteins than conventional techniques. In some cases, a normally glycosylated protein is active without the carbohydrate moiety and can be made in bacteria. This is the case with certain interferons. In cases where glycosylation is ncessary, this can often be provided by recombinant fungi, insect or mammalian cells.

In addition to natural polypeptides, analogs can be produced by protein engineering and this has added an extra dimension of excitement to the field: (1) hybrid proteins can be produced by in vitro recombination of hybrid genes, e.g., hybrid interferons have proper-ties different from the individual interferons; (2) analog polypeptides can be produced by chemical synthesis of DNA containing triplets encoding a different amino acid than is present in the natural polypeptides; (3) a cloned gene can be randomly mutagenized before insertion into the vector; (4) site-specific mutagenesis can be employed using DNA polymerase to prepare mutant synthetic oligonucleotide primers on single-stranded DNA templates; and (5) deletions or insertions of a few nucleotides in length can be carried out using restriction enzymes and genes can be truncated by insertion of stop codons.

Analogs will be especially useful to perturb human bioregulatory systems in the area of pharmacology. The pharmaceutical industry is using rDNA techniques to discover the sequence of bioregulatory proteins and peptides and to prepare analogs to test for receptor binding and effector activity. The final step in this new pharmacology is to synthetically (via computer graphics) design stable possibly non-peptide mimics of the active sites of the natural molecules or their analogs.

Vaccine production is a major part of the new technology. These protein antigens can be made by cloning and expressing genes coding for viral, bacterial and parasite surface antigens. The first approved human subunit vaccine is hepatitis B virus surface antigen which is produced commercially in yeast. Of recent importance is the cloning of the major surface protein of the sporozoite of the human malarial parasite, Plasmodium falciparium, which could lead to a vaccine against malaria.

Another area being developed is the cell-free (enzymatic) synthesis of antibiotics (19). Cloning, expressing and immobilizing antibiotic synthetases could lower the cost of semi-synthetic antibiotics and yield novel antibiotics.

The future is thus insured for the expanded use of microorganisms in the pharmaceutical world and the continued improvement in microbial processes to reduce the cost of such drugs.

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