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RECOMBINANT DNA TECHNOLOGY I

Edited by Aleš Prokop and Rakesh K. Bajpai

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RECOMBINANT DNA TECHNOLOGY I[a]

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Prospectives and Challenges in Genetic Engineering

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Genetic engineering is the technology by which man chooses and selectively breeds mutants in order to use the resulting organisms for his own benefit. This enterprise started with the first domestication of animals and plants or, perhaps, with the first maintenance and use of microorganisms for food and beverage processing. Although the associated technology and the uses of genetic engineering have changed dramatically since the early 1970s, it is important to realize that genetic engineering is not a new endeavor.

During the last decade we have seen an explosion in the use of (and interest in) genetic engineering. This is largely the result of two developments: the basic scientific discoveries of the structure and functioning of DNA (what genes are, how they are expressed, and how the expression is regulated), and the development of new techniques for analyzing and manipulating DNA molecules (and other biological macromolecules). Although particular research accomplishments can be pointed out as highlights in these developments, the whole process was, as I shall attempt to point out, the cumulative result of the efforts of many scientific projects, the great majority of which were not designed to aid in the development of this enterprise.

Several basic science discoveries were critical in the development of the new technology. Perhaps the first critical findings were that DNA was the genetic material and that DNA acted, for the most part, by encoding the primary sequence of proteins.[1-3] This led to work that elucidated (1) the mechanisms by which this information transfer occurs (usually DNA to RNA to protein),[4] (2) the nature of the code that relates nucleotide sequence to amino acid sequence,[5] and (3) the controlling mechanisms that define individual units of expression and regulate their activity.[6] Of course, the motivation for much of this work may not have been to develop the technology of genetic engineering; rather, it was an attempt to understand life and, for some scientists, it was for personal enjoyment and aesthetic satisfaction. In addition, many discoveries that were made should have a basic impact on our philosophical systems. (What is the real consequence of discovering that life is governed by biochemical reactions?[7] The ubiquitous existence of transposable elements[8] implies that perhaps a basic level of evolution is "competition" between DNA sequences and that each organism carries within its own genome the instructions for changing that genome. The discovery of introns[9,10] has challenged, for awhile, the notion that nature tends toward greater economy.) Nonetheless, the discoveries have given us guidelines that we as "engineers" can use to plan the alteration of selected organisms in specific ways. For instance, if we wish to have an organism make a particular protein, we now know what the structure of the gene should be and what signals are needed for controlling its synthesis.

Knowledge is not enough. Engineers also need tools to apply the knowledge. Although my list may be exhausting, it will not be exhaustive. Of critical importance

was the development of the field of nucleic acid enzymology. Many point to the discovery of restriction enzymes[11] as a watershed event, because it enabled us to dissect DNA molecules into defined fragments. However, being able to fragment DNA molecules without knowing how to link various fragments together would not have been very useful; therefore, the discovery and analysis of DNA ligases[12] were important. Likewise, due to the efforts of many unheralded scientists, we have enzymes that will degrade DNA, synthesize DNA, and phosphorylate and dephosphorylate the ends of polynucleotides, and so forth.[13] Other current techniques required the development of DNA sequencing methodologies,[14,15] chemical techniques for synthesizing DNA,[16] and methods for purifying and handling various macromolecules. Most of the methodologies also make use of basic microbiological protocols as well as the discoveries associated with bacteriophages, plasmids, and so on.

Which technologies will have a particularly big impact in the next 10–20 years? My three favorites are: (1) nucleic acid hybridization technology as a diagnostic tool, (2) antisense RNAs or ribozymes as pharmaceuticals, and (3) designer monoclonal antibodies made in *Escherichia coli.*

The use of nucleic acid hybridization technology[17] to identify the presence of specific DNA sequences (and consequently the presence of specific organisms or specific rearranged genes in a given organism) is commonplace in the research laboratory. We already know its use in the forensic laboratory and in paternity testing. The only questions are not if, but rather, why has this technology not been used already, when will it be used, and what detection modalities will be used.

Antisense RNAs[18] and/or ribozymes[19] block gene expression through the same molecular principle, that is, sequence-specific nucleic acid hybridization. Just one example suffices to show its potential usefulness. Attacking a virus during an infection process is problematical because the host cell can be killed as well. In principle, these techniques eliminate this problem by virtue of their specificity. Although these techniques can work right now in the research laboratory, the jump to a practical pharmaceutical application may not be simple. We are faced with major questions of cell targeting and efficient introduction into the cell.

Finally, designer antibodies. Antibodies have the fantastic ability to recognize and bind to specific molecular structures. The potential applications are endless. With the recent development of *E. coli* systems for producing such monoclonal antibodies,[20,21] we now can envision inexpensive and abundant sources for any antibody we wish.

If current controversies and newspaper headlines are any measure, the public is equivocal about accepting modern genetic engineering as a productive technology. In a sense, this is paradoxical, because almost all of our food is a direct product of genetic engineering, and the new technologies are likely to provide, if anything, a more controlled application of genetics. My guess is that our school systems do not educate (and have not educated) our children adequately in genetics (How many people really know that biological properties are inherited?) or in biochemistry (Are life processes really controlled by chemical reactions?). The problem may reflect a general need for upgrading K through 12, but I worry that it may also reflect the fact that the science of genetics and biochemistry challenges some generally accepted philosophical norms. After all, is it not the basis for the creation-evolution controversy? Perhaps there is an almost deliberate wish by our educators to ignore these sciences. When one takes this perspective and adds in a distrust for high technology, is it surprising that the public might be suspicious when genetics and biochemistry are used so obviously? I think not. My only solution is that the scientific and industrial community must start taking education more seriously. A very important

mechanism by which the private sector can further public education is through judicious product selection. The choice of products that address perceived needs is likely to encourage a higher comfort level with the technology. An important part of this process should be public safety concerns.

My final concern has to do with the fiscal health of the academic research community. The current situation is guarded and the prognosis is not good. Almost all academic scientists believe that the key to a healthy academic research community is the individual investigator-initiated research grant system. In NIH terminology, these are RO1 grants. The situation is close to desperate. Although the stated numbers vary (and this is symptomatic of one of the problems), the huge majority of competing grant proposals, including the majority of competitive renewal proposals, are not being funded. Why is this important? Without a healthy grant system, most academic research will cease, most graduate student and postdoctoral training will cease, and most undergraduates will get the message that scientific careers are not viable. This is at a time when 30% of our faculties will retire in the next 10–15 years.

How will this crisis affect the biotechnology industry? In the short run, it will probably be an asset. Talented scientists will be encouraged to drop out of academia, and those that stay will seek industrial ties. But in the long run, it will be a disaster. Five years from now, the employee pipeline will start to dry up. In addition, almost all of the basic scientific discoveries and technological developments described before were the products of individual investigator-initiated research projects. We will need new discoveries, but the research will not have been done.

What can we do? I suggest that two initiatives are needed. Number one, increase funding of individual investigator academic research. Little more needs to be said than "give me a Stealth bomber and help me to provide employment to technical help, secretaries, dishwashers, etc." Number two, let's get some leadership at NIH and let's have NIH (and Congress, if necessary) review its priorities. NIH first has to admit that there is a crisis. I haven't heard it yet. Second, NIH has to review the cost and validity of high indirect costs and of completely funding some faculty salaries. Let's have some good old-fashioned decisions about what NIH is supposed to be doing. Third, NIH has to realize that behind big science (e.g., the genome initiative) is small science—good old-fashioned molecular genetics. Finally, NIH has to have an equal playing field, that is, the same quality standards for large targeted grants as for individual grants.

Let me close by being honest. We have a potentially bright future, but if we are going to realize it, we need private industry's help, help in education, help in research funding, and help in lobbying.

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Role Played by International Meetings of Genetics of Industrial Microorganisms

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The late 1960s was a time when antibiotic-producing strains, especially tetracycline, were important and much work was done, mostly in industrial laboratories, to improve the technology, strain productivity, and biochemistry of compounds. Mutagenesis was used to improve strains, and auxotrophic and nonproductive blocked mutants were isolated, both techniques helping a great deal to determine the biosynthetic pathways of desired metabolites. Furthermore, marked strains were used to construct genome maps at a time when little was known about either the mechanisms of mutagenesis of different mutagens or the repair of DNA lesions.

Prof. Giuseppe Sermonti, who helped me to learn the different methods available, and I discussed at length the necessity of establishing more direct contact and exchange of experience between scientists in industry and those in academic laboratories. Because virtually no contact existed between scientists from academic institutions and those from industrial laboratories, we decided to organize an international meeting on *streptomycetes* and antibiotics, the most interesting industrial subjects at that time. The meeting in Dubrovnik, Yugoslavia, took place from May 31 to June 2, 1968. I would like to quote from remarks made by the President of the Meeting, Prof. Sermonti, and its Chairman, Prof. Hopwood.

> After ten years we can make a first balance of the progress achieved both in the genetics and the breeding of *Streptomyces*. Pure genetics of *Streptomyces* is no longer in its infancy and has made considerable advancements mainly thanks to the work of the Glasgow group. The biochemistry of antibiotic biosynthesis by *Streptomyces* has also recorded great achievements, especially in the field of the tetracyclines. Considerable success has been made in the breeding of various antibiotic-producing *streptomycetes*, although I must admit that I am only vaguely informed about them.
>
> There has been, in general, a lack of osmosis between theoretical genetics and practical microbiology. It must be said that Dr. M. Demerec was an exception to this rule, having been the first to introduce the mutagenic treatment in the selection of high rated strains of Penicillium.
>
> Interesting attempts to fill the gap will be reported in this meeting, and I hope that some relevant information will emerge from the discussion. It is the chief aim for this Symposium to develop a fruitful dialogue between the two sides of the field, the theoretical and the practical. It would thus be desirable if the speakers dealing with pure genetics and biochemistry could emphasize the possible practical implications of their findings, and, vice versa, if the speakers dealing with breeding could put emphasis on the problems that they think would require a more refined theoretical approach.
>
> (Giuseppe Sermonti)

> This morning's session is devoted almost exclusively to a particular strain, A3(2), of *Streptomyces coelicolor*. This is, of course, not an antibiotic-producer, and has no known industrially interesting attributes. It simply happens to be the organism with which most of the work on streptomycete genetics has been done. Clearly it would be inefficient to

switch the major effort at this stage to some strain of typical industrial interest, which may not even be sustained. It is surely better to concentrate the main effort in "academic" research on A3(2)—just as most of the work on the genetics of enteric bacteria has been done with *Escherichia coli* K12 and *Salmonella typhimurium* LT2—but at the same time to compare genetic phenomena in other streptomyces with the A3(2) model to see to what extent techniques have to be modified to succeed with other strains. Some of the contributions later in this meeting will illustrate this approach.

(D. A. Hopwood)

We now know that many essential differences exist among *Streptomyces,* especially their productive strains, such as differences in map arrangement (empty zones); the role of different parts of the genome; gene amplification and instability; plasmid structure and stability; interspecific recombination; and the position of genes included in antibiotic biosynthesis. On the other hand, much data show a great similarity between parts of the genome included in primary metabolism that have led to different speculations on the development of the *Streptomyces* genome.

At the Dubrovnik meeting, as cited by the President and the Chairman, a significant advance in the research of radiation and chemical mutagenesis was shown as well as an already well-defined map of *S. coelicolor,* much data on the mutation and selection of productive strains and screening programs from different laboratories, and recombination processes, genetic analysis, and mapping of genomes of *Streptomyces,* primarily *S. coelicolor.* Also discussed were the mutation in *Streptomyces* from research into the effects of radiation and chemical agents on DNA, the possibility of selecting better producers in different screening programs, and the role of block mutants in the biosynthesis of tetracyclines (chlortetracycline). The study of actinophages was also presented in one lecture.

At this meeting a group of scientists, including Sermonti, Hopwood, Alikhanian, McCormick, Vanek, and myself, discussed the necessity of organizing regular meetings on the genetics of industrial microorganisms with the aim of improving collaboration between academic and industrial scientists.

The first general international meeting was organized in Prague in 1970. Vanek, in his review of the meeting, remarked that throughout the decades microbiology and genetics have been the foundation for the great development of the fermentation industry which not only has produced extremely valuable drugs and chemicals but has also become important in the production of food feed and in solving ecological problems.

With progress in the antibiotic industry and with more funds for basic research into molecular biology and formal genetics, great advances occurred in our knowledge through joint research.

The most characteristic feature of industrial microbiology was the proliferation of studies into molecular genetics, the results of which brought us to the fundamentals of biochemical and genetic control of microbial metabolism. This gave rise to what was to be called molecular biology and the newest methods for tailoring and constructing new strains. The rapid development of industrial microbiology and genetics proceeded almost independently, although their basis as well as their results led to similar ends. The start was determined by the work of industrial microbiologists, whereas genetics remained almost entirely basic and theoretically oriented. Those working with industrial microorganisms were to profit from a knowledge of genetics and new methods, acquiring a rationale for their work and hence intensifying their search for less empirical but quicker results for improving their strains. Geneticists were able to refocus their research from formal genetics and the control of primary metabolic pathways to the level of formation of special products and to extend the range of models for research. The mutually coupled investigation in this

direction thus opened the way for other contributions of microbiology in solving problems of fundamental biology, for example.

In other words, only a complete understanding of the physiological metabolic and genetic laws could help uncover how and why different products are formed in an organism during cell differentiation. The subjects of the Prague meeting were similar to those of the Dubrovnik meeting, but the number of presentations and participants and the breadth of knowledge were greater.

To achieve excess production of the desired compound, industrial scientists invented many ingenious tricks for overcoming the regulatory mechanisms. This demonstrated that industry employed outstanding scientists who could convert the basic research data to practical ends in a remarkably short time.

There are many examples to document that through the use of fundamental knowledge in strain selection, backward pressure was exerted on pure science, stimulating the solution of further questions of fundamental importance. Beadle and Tatum used the "industrially unsuitable" model of *Neurospora crassa* to study the "industrially uninteresting" auxotrophy that later became the basis of molecular biology, and Kellner first observed photoreactivation in *S. griseus* while working on an industrially interesting strain that explained one of the repair processes in cells.

The whole area, however, was mutually criticized. Doubt arose concerning the suitability of model organisms that were studied, and feedback from industrial microbiologists was almost completely lacking.

The first indisputable achievement of the Prague symposium was that the intended bringing together of "applied" microbiologists with those scientists who contributed to our knowledge of genetic regulation was fully appreciated. This made it possible to confront our present fundamental knowledge with the needs of practical industrial application.

The goal of bringing together the theoretical and the practical scientists at the Prague meeting was achieved, but how was the collaboration to proceed? This problem was very neatly addressed in the final lecture given by A. L. Demain entitled, "Marriage of Genetics and Industrial Microbiology—After a Long Engagement, a Bright Future." Demain was not sure when the bright future would start: "The partners have not yet fully committed themselves to working toward the mutual benefit of the pair, each going down a separate path to a considerable extent. If the union is ever consummated, we can look forward to a bright future for both."

Vanek concluded that it would be immodest to expect miracles from a meeting of this type; still, it cannot be overlooked that the symposium succeeded in focusing attention on theoretical problems, defining fields of future study and indicating some methodical approaches to be used. It was, above all, obvious that a successful solution would require a complex combined approach by geneticists, physiologists, enzymologists, chemists, bioengineers, and physicists. The conclusion that the symposium would represent just the beginning of a regular series of symposia was unanimously accepted.

Simplicity of the mutation techniques and the ease with which microorganisms could be changed by mutation attracted industrial microbiologists and managers, and extensive programs of mutation and selection in strain improvement still exist today in industrial laboratories throughout the world. Fifty years later, it is clear that mutation has been the major factor in up to a thousandfold increase in the production of microbial metabolites, without there being much knowledge about the mechanisms of their action. Today, at a time when geneticists are faced with possibilities unimaginable 50 years ago, we still need to rely occasionally on the old methods, especially selective ones.

Unfortunately, the randomly selected mutants used to increase the production in

industry were less attractive for geneticists, and although changes in colonial and cellular morphology were very useful in the selection of active strains, there was no real understanding of what was happening in the cell and colony.

A combined approach based on a knowledge of fundamental research and mutagenesis, life cycle, and physiology presents us with the best approach to strain improvement.

To illustrate this approach, in the selection of alkaloid-producing strains we used strains that were totally nonproductive in a submerged culture but productive in the parasitic stage. First, we carefully studied the cellular and mycelial morphology of the parasitic strain in its productive phase and then we used several mutagens with known mechanisms and conditions. We cultivated the mutated cells together to allow contact of survivals to form multinucleate or diploid cells as they are in sclerotial phase (productive phase) in the parasitic strain. The result was excellent and quick.

To choose the best mutagen, a knowledge of the mechanisms of mutagenesis was needed. Particularly interesting was the discovery that mutation methodology could be used to produce entirely new molecules. A further use of mutants was to elucidate secondary metabolic pathways. The isolation of a good producer, however, has raised many questions, and numerous scientists have tried to explain what really happens in the cell despite many years of observing the phenomenon and its successful use in production.

The use of genetic recombination to improve yields of fermentation products was disappointing, despite the fact that recombination is supposed to be the major source of genetic variation and that many types of recombination occur in microorganisms. The failure of classical recombination with the exchange of large parts of the genome in strain improvement may be explained genetically by the instability of diploids, the recessive nature of genes that lead to superior production, and the lack of methods to select the desired high-producing recombinants. The results show that mutation is the source of the variability and that natural recombination is the way of stabilizing the natural relationship between features in the cell and its existing environment.

Presumably because of the need to maintain chromosomal integrity, recombination *in vivo* requires nearly perfect homology and is abolished by as little as 10% sequence divergence. This may influence the recombination frequency between divergent strains. The mismatch repair system might prevent recombination between partially homologous sequences and provide a functional barrier to interspecies recombination. The authors have now tested the prediction that mutants defective in mismatch repair can become proficient in interspecies recombination by using members of two related bacterial genera, *E. coli* and *S. typhimurium,* which are about 80% homologous in the DNA sequence. Mutations in the *mutL, mutS,* or *mutH* mutator genes inactivate methyl-directed mismatch repair of errors in DNA replication. Rayssiquier *et al.* showed that these mutations can increase intergeneric recombination in conjugational and transductional crosses up to a thousandfold. Hence, mismatch recognition and the mismatch repair system can act as a barrier to recombination between DNAs of different species. The use of technologies that allow interspecific recombination may be wide ranging in future biotechnology.

At the Sheffield meeting of Genetics of Industrial Microorganisms (GIM) in 1974, Pontecorvo stated:

> One thing is clear to the outsider. The advances in the applications of genetics to the improvement of strains of industrial microorganisms are trifles compared to the advances in the fundamental genetics of microorganisms.
>
> With a general basis of knowledge and techniques as formidable as those in microbial genetics it is very disappointing to see how far behind are the applications to the improvement of industrial microorganisms.

> The main technique used is still a prehistoric one: mutation and selection. In 1940 independently Demerec, in the USA, and myself in Scotland proposed this technique only as a war-time emergency measure for improving penicillin yields. Penicillin was desperately needed then and even an approach intellectually crude and, a priori, not very likely to be successful was worth a trial. The success was so unexpectedly good that, unfortunately, since then most industrial laboratories have been contented with its exclusive use.
>
> Sexual reproduction, combined with diploidy, is the most highly elaborate of these mechanisms. It is at least 25 years since Müller showed in a simple graphic way how mutation and selection by themselves are so much less effective than in combination with processes of transfer of genetic information.
>
> If industrial laboratories were doing successfully something along these lines, we would not hear about it.

Why does this enormous gap exist between basic knowledge and its application? Pontecorvo mentioned two reasons: fragmentation of effort and predominance of chemical outlook in the microbiological industries. Let me deal with this second point first. Most of the fermentation industries are offshoots of the chemical and, more particularly, pharmaceutical industries. Naturally in these mother industries the predominant outlook was that of the organic chemist. This idea was carried over into the fermentation industries, later only partially infiltrated by biochemistry.

The second reason for the disappointing state of the application of genetics to the improvement of industrial microorganisms is the fragmentation of effort. Every industrial concern has its own miniteam working in secrecy and trying to produce more desirable strains. The absurdity of this situation can be illustrated by an analogy: "agriculture in Great Britain is a highly fragmented, privately run industry vastly more important than all the microbiological industries taken together. Yet, the improvement of varieties of crop plants, which was mainly an individual concern up to a century ago, is now concentrated in three main plant-breeding stations. They are quite successful and the farming community seems to be happy about them. No farmer in his senses, no matter how large his farm, would dream of breeding his own varieties of crop plant and, in addition, keeping them jealously for himself. Recruitment of first class geneticists to the plant-breeding stations was no problem at all. The charge that academic geneticists are reluctant to turn to full-time applied work, quite correct in the case of the microbiological industries, is wrong in the case of agriculture. What should we do about all this? The problem is how to have industrial plants and know-how directly connected with teams of first-class geneticists working on strain improvement. In analogy to plant breeding, this improvement can be carried out efficiently only in a few highly specialized centres."

"I realize that I have dropped a few huge bricks, but I hope they will lead to a serious reappraisal of the situation," Pontecorvo concluded.

Pontecorvo's words coincided with the explosion of new knowledge, after which closer relations between the two unexpected partners began to develop. However, the problem was much more profound than the foregoing technical difficulties indicate. Given that more was known about the mechanisms of mutagenesis and recombination, the approach to strain improvement through classical recombination could have been more successful. Progress in microbial specialty molecular genetics over the last 30 years was truly fantastic and it has shown how knowledge may be directly applied when conditions on both sides are ripe. I believe that this signaled the long-awaited marriage that Demain had referred to earlier.

It has now been demonstrated that the division in industrial or academic strains is

no longer pertinent. The most academic strain, *E. coli,* has become industrial and every industrial species, such as yeast and some *Streptomyces,* has become academic.

Although the two GIMs of 1974 and 1978 dealt with similar problems, it was evident that the accumulation of basic knowledge was about to explode with something new. The Kioto meeting, with its subjects or rather its focus on methods used, was totally different from the previous ones.

In vitro genetic engineering of industrial microorganisms, which was the main subject in both Kioto and Split meetings, stems from a vast knowledge of *in vivo* genetics of *E. coli* K-12 and its phages and plasmids. The applications of gene cloning in *E. coli* include natural genetic phenomena as well as unnatural ones. Other "academic" strains, such as *Bacillus subtilis* and *Saccharomyces cerevisiae,* also were immediately included in new research and became important hosts for recombinant DNA because they were well established in other genetic work. In *Streptomyces* strains the situation was slightly different because *S. lividans,* instead of *S. coelicolor,* became the first experimental organism in genetic engineering. Protoplast fusion, alone, although unsuitable for fundamental research, was useful in the selection of some types of production, and naturally the knowledge of methods for protoplast and mass regeneration helped in recent DNA experiments.

The importance of traditional mutation and recombination methods for improving industrial strains was emphasized in the panel discussion convened by the chairman of GIM. Progress in these methods as well as in the techniques of site-directed mutagenesis and chemical synthesis of DNA was referred to frequently in many presentations.

The GIM International Symposia have become very important in the field of biotechnology, and the topics reported at GIM-82 and GIM-86 drew great attention worldwide.

The last 20 years have shown the dramatic development of new methods for effecting genetic changes in industrial microorganisms. We are already applying all these methods. Their more useful results, however, will come from combinations of different procedures. We can now obtain desirable new combinations of nonhomologous DNA segments from the two species, and with a sufficiently powerful selection, we can hope to produce a complex new genotype that cannot be constructed rationally because its components cannot be identified in advance.

We are indeed living in an exciting time for the genetics of industrial microorganisms with its endless possibilities. The close collaboration between academic and industrial microobiologists has taken a long time to be realized. Pontecorvo, however, would be gratified to witness the proliferation of highly specialized centers like those he admiringly made reference to in his plant-breeding analogy.

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