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# GENETICS AND MICROBIOLOGY OF INDUSTRIAL MICROORGANISMS

## Molecular genetics and industrial microbiology — 30 years of marriage

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**Thirty years ago, molecular genetics and industrial microbiology joined their hands in marriage. The event took place in Prague at the first Symposium on the Genetics of Industrial Microorganisms. My closing plenary lecture, titled "The Marriage of Genetics and Industrial Microbiology — After a long Engagement, a Bright Future," dealt with industrial uses of mutants, the lack of success with genetic recombination, control of branched and unbranched pathways and thoughts about the future, e.g., identifying the biochemical sites of beneficial mutations, exploitation of recombination and genetic means to increase production of enzymes. It is quite amazing that the Symposium was held 3 years before the advent of recombinant DNA technology. This important meeting was followed in 1976 by the first Genetics and Molecular Biology of Industrial Microorganisms (GMBIM) meeting in Orlando, all of the six subsequent GMBIM meetings being held in Bloomington, Indiana. Today, thousands of biotechnology companies are in existence making great progress in the pharmaceutical and agricultural sectors. Hundreds of new genetically engineered compounds, produced in microbial, mammalian or insect cells, are in clinical trails and many are already being marketed. The field is booming with new technologies such as transgenic animals and plants, site-directed mutagenesis, combinatorial biosynthesis, gene therapy, antisense, abzymes, high-throughput screening, monoclonal antibodies, PCR and many more. Agricultural biotechnology has made great strides but unfortunately its progress is being delayed by political controversy.** *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 352–356.

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Industrial microbiology has been around for a long time. Since pre-biblical times, microorganisms have been making products such as beer, wine, cheese, vinegar and pickles, to preserve our milk, fruits and vegetables and serve our palate. In the 20th century, a golden age of industrial microbiology yielded a myriad of products made by fermentation processes: solvents, antibiotics, enzymes, vitamins, amino acids, flavor nucleotides, polymers, bioconverted steroids and other useful compounds [11]. Thirty years ago, molecular genetics and industrial microbiology came together. The event took place in 1970 in Prague at the first international symposium on the Genetics of Industrial Microorganisms (GIM). My closing plenary lecture, titled "The Marriage of Genetics and Industrial Microbiology — After a Long Engagement, a Bright Future," dealt with the industrial uses of mutants, lack of success of genetic recombination for strain improvement, control of branched and unbranched pathways and thoughts about the future, i.e., identification of the biochemical sites of beneficial mutations, exploitation of recombination and genetic means to increase production of enzymes. It is quite amazing that this symposium was held 3 years before the advent of recombinant DNA technology. GIM meetings have taken place every fourth year since then in various cities around the globe. In 1976, Harlyn Halvorson and I organized the first Symposium on Genetics and Molecular Biology of Industrial Microorganisms (GMBIM) in Orlando. All of the six subsequent GMBIM meetings have been held in Bloomington, Indiana.

The scientific revolution that occurred in the early 1970s launched modern biotechnology. It was brought about by the discovery of recombinant DNA by Paul Berg and Stanley Cohen of Stanford University and Herbert Boyer of the University of California, San Francisco in 1972–1973. This development did not occur in a vacuum but was a logical development after many years of highly significant genetic discoveries (Table 1).

The biopharmaceutical industry began in 1971 with the establishment of Cetus in Berkeley [38]. In 1975, the first monoclonal antibodies were produced and 1976 featured the founding of Genentech, the introduction of DNA sequencing and the replication and expression of yeast DNA. In the next year, a human gene was expressed in bacteria and the rat insulin gene was cloned. Biogen was founded in 1978, the same year that recombinant human insulin and human growth hormone were produced and bacterial DNA was successfully inserted into yeast chromosomes. In 1979, yeast protoplasts were transformed by a hybrid *Escherichia coli*/yeast plasmid and 1980 saw the founding of Amgen and the ruling by the US Supreme Court that living organisms could be patented. In 1981, the first recombinant diagnostic kit was approved by the FDA and three more companies were formed: Genetics Institute, Chiron and Genzyme. Recombinant human insulin was

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**Table 1** Some milestones in genetics up to 1970

| Year | Milestone |
|---|---|
| Mid 19th century | Mendel — characteristics in peas are inherited |
| 1941 | Beadle and Tatum — one gene, one enzyme concept |
| 1944 | Avery, MacLeod and McCarty — DNA is genetic material |
| 1946 | Lederberg and Tatum — bacteria have sex |
| 1950 | Chargaff — base pairing in DNA |
| 1953 | Watson and Crick — DNA exists as double helix |
| 1953 | Hayes — conjugation, fertility plasmid, Hfr strains |
| 1961 | Monod and Jacob — regulation of gene expression |
| 1962 | Smith, Arbor — restriction endonucleases |
| 1966 | Nirenberg, Matthaei, Leder, Khorana, Ochoa — genetic code deciphered |
| 1969 | Shapiro and Beckwith — isolation of a gene |
| 1970 | Khorana — chemical synthesis of a gene |

approved in 1982. In 1984, the HIV genome was cloned and sequenced by Chiron. One year later, human growth hormone was approved and Cetus announced the development of polymerase chain reaction (PCR) technology. In 1986, $\alpha$-interferon and the first recombinant vaccine for hepatitis B were approved and in 1987, approval was given to tissue plasminogen activator (tPA). The human genome project was begun in 1988, the year that the first patent on transgenic animals was issued. Erythropoietin (EPO) was approved in 1989 and 1 year later, human gene therapy was attempted for the first time. In 1991, granulocyte colony-stimulating factor (G-CSF) was approved and in 1992, approval was given to Factor VIII. $\beta$-Interferon was approved 1 year later.

Recombinant DNA technology found its way into areas of primary metabolism. For example, *E. coli* was converted from a producer of mixed acids into a producer of acetone [3] by introduction of the acetone operon from *Clostridium acetobutylicum*. The operon included *ade* (acetolactate decarboxylase), *ctfAB* (coenzyme A transferase) and *thl* (thiolase). The recombinant *E. coli* was found to produce 9 g of acetone per liter, more than the *C. acetobutylicum* donor. *E. coli* was also made into a good ethanol producer forming 4.3% (v/v) ethanol [19]. Genes encoding alcohol dehydrogenase II and pyruvate decarboxylase from *Zymomonas mobilis* were inserted into *E. coli* and became the dominant system for regeneration of NAD.

The concept of metabolic engineering was born out of many failed attempts by geneticists to improve titers and/or yield of microbial processes by cloning and expressing pathway biosynthetic genes. Biochemical engineers felt that they could help choose the correct gene to overexpress by determining limiting enzyme(s) *via* analysis of flux through the metabolic pathway by any of the following techniques: (I) kinetic-based models, (ii) control theory, (iii) tracer experiments, (iv) magnetization transfer, (v) metabolite balancing, (vi) enzyme analysis, or (vii) genetic analysis [14]. Flux analysis has led to some success in improvement of amino acid and antibiotic production processes by the use of recombinant DNA technology. The regulatory controls on secondary metabolism are numerous and complex and include the following [8]: cell density, pheromones, sensors, growth rate, nutrient limitation, low molecular weight effectors, metabolic imbalance, stress responses, nutritional induction, repression and inhibition, pleiotropic regulatory genes and pathway-specific regulatory genes. An understanding of the relationships between these factors will aid in metabolic engineering.

Genetic analysis was crucially important for the discovery that the antibiotic erythromycin is formed in *Saccharopolyspora erythraea* by a polyketide synthase (PKS) system that is modular in nature, containing multidomain enzymes [9,12]. Six modules were found, each containing several catalytic domains. Each module is responsible for addition of one three-carbon unit from methylmalonyl CoA to a growing chain started with propionyl CoA. Whether or not a module contains a ketoreductase, dehydratase and an enoyl reductase activity determines the extent of processing of the $\beta$-carbonyl of the growing chain, i.e., its level of oxidation. Similar modular pathways were found for rapamycin and rifamycin biosynthesis and for many other nonaromatic polyketides. After the discovery of the modular nature of erythromycin biosynthesis, it was found that deletion or disruption of a gene in the pathway could lead to the production of a new polyketide derivative of one of the bound intermediates in the biosynthetic scheme. Similar disruptions in the biosynthetic PKSs of type II polyketides, i.e., aromatic polyketides, also yielded new compounds. Furthermore, it was shown that interchanging PKS genes among producers of different polyketides could yield novel polyketides [17]. This concept of combinatorial biosynthesis has led to the creation of hundreds of new metabolites, some of which are shown in Figure 1 [22]. Similar manipulations have been done with peptide antibiotics [20]. It is hoped that some of these novel products will have useful properties suitable for pharmaceutical development.

A major part of the biopharmaceutical effort has been the choice of the proper host for recombinant DNA. It was logical that *E. coli* would be chosen first because it had been the subject of years of biochemical and genetic study. Table 2 shows the reasons for the successful application of *E. coli* [37]. Other unicellular bacteria that have been used for cloning include *Bacillus subtilis*, *Bacillus brevis* and *Bacillus megaterium*. The titers of mammalian peptides produced by unicellular bacteria have ranged from 10 mg to 12 g per liter.

*Saccharomyces cerevisiae* has also served as a frequent host because it has a long history in industrial fermentation, can secrete heterologous proteins into extracellular broth and can glycosylate proteins, unlike unicellular bacteria. Furthermore, eukaryotic cells can carry out other posttranslational modifications such as addition of fatty acid chains, and phosphorylation of tyrosine, threonine and serine hydroxyl groups. The presence of sugar on the molecule is important to certain proteins for *in vivo* activity, immunogenicity, reaction kinetics, receptor binding, serum half-life, solubility and/or thermal stability. However, in certain cases, *S. cerevisiae* overglycosylates the recombinant protein. In such cases, the methanol-utilizing (methylotrophic) yeasts *Pichia pastoris* and *Hansenula polymorpha* have been used. These can be grown to higher cell densities, have higher protein productivities, and can integrate multiple copies of foreign DNA into their chromosomes.

Bacteria and yeasts have been subject to biochemical engineering studies to achieve very high cell densities. Thus, even with low levels of gene expression, the titer of product can be high. For bacteria, dry cell weight has reached over 230 g/l whereas with yeasts, the figure is almost 270 g/l [33]. Filamentous fungi such as *Aspergillus* sp. have been used to a limited extent for production of recombinant proteins.

In many cases, recombinant proteins are produced in higher eukaryotes such as insect cell cultures. Over 200 proteins have been produced in hosts such as the fall armyworm in suspension or larval

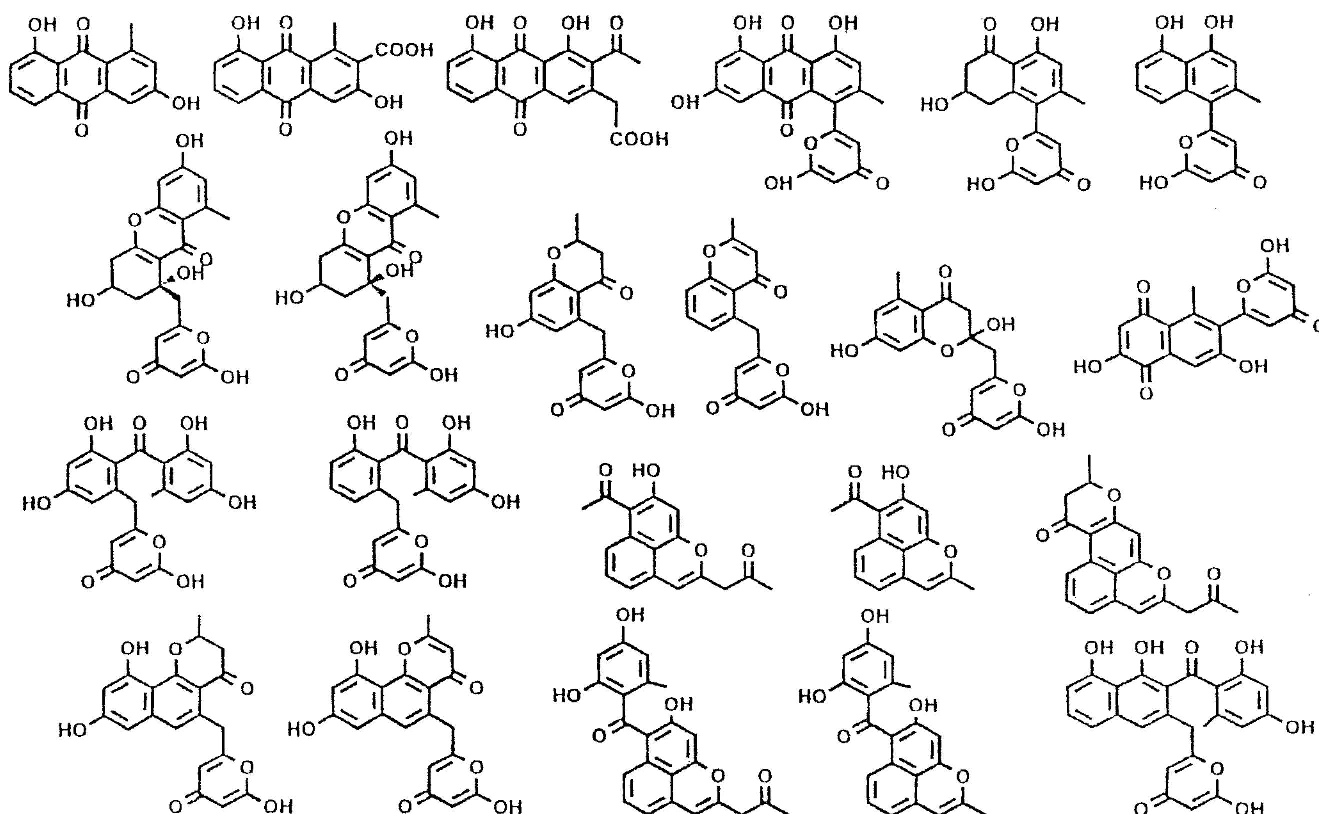

**Figure 1** Novel polyketides produced by mutated *pks* gene clusters from the producers of actinorhodin, frenolicin and tetracenomycin [22].

culture. The usual vector is a baculovirus such as the nuclear polyhydrosis virus and the promoter used is that for polyhedrin biosynthesis. These baculoviruses are not infectious to vertebrates or plants. The proteins made in insect cell systems often contain the same types of posttranslational modification as their natural counterparts such as proper phosphorylation, glycosylation, signal peptide cleavage, proteolytic processing, palmitylation and myristylation [24,27].

In cases where incorrect glycosylation occurs (e.g., sometimes with yeasts and insect cell cultures), mammalian cells are employed for protein production [4]. Work started on such systems in the early 1980s when it was found that *E. coli* could not produce glycosylated EPO or tPA. The chief host cell used has been the immortalized Chinese hamster ovary (CHO) cell. In 1997, the market for biotherapeutics produced in mammalian cells was $3.25 billion whereas that for *E. coli* was $2.85 billion [23].

In recent years, the milk of animals has been shown to be an attractive system for producing recombinant proteins [13]. Indeed, human hemoglobin has been produced in pigs at 40 g/l, human $\alpha$-1 antitrypsin in sheep at 35 g/l, recombinant fibrinogen in sheep at 5 g/l and human growth hormone at 4 g/l in mice. Both antithrombin III and $\alpha$1-antitrypsin are in late-stage clinical trials with no adverse reactions being reported yet [26]. The urine of animals has also been claimed to be a useful site for recombinant protein production [21]. Transgenic plants are also being developed as hosts. Oilseed rape has been engineered to produce enkephalin and a neuropeptide whereas polyhydroxybutyrate has been produced in *Arabidopsis*.

The biopharmaceutical business is booming today in hundreds of small biotechnology companies and large pharmaceutical and chemical corporations. Hundreds of new genetically engineered compounds are in clinical trials and many are already being marketed. Several products exceed sales of over $1 billion per year. The leader is EPO, which has worldwide sales of $4 billion. In 1998, 54 biopharmaceuticals had made it to the market [25]. In 1999, the US biotechnology industry had 900 companies, over 150,000 employees, revenues of $20 billion and research and development expenditures of $11 billion [39]. Up through 1999, approximately 100 products were on the market in the biopharmaceutical and agricultural areas. Interesting new products include DNA vaccines, key DNA fragments from pathogens, which, upon injection, induce human cells to produce the encoded protein [6]. A growing number of monoclonal antibodies have been approved for treatment of cancer, Crohn's disease and viral infections [16,18].

**Table 2** Reasons for use of *Escherichia coli* as a recombinant host [37]

1. Quick and precise methods to modify its genome
2. Rapid growth
3. Ease of culture
5. Ease of protease reduction
6. Ease of avoidance of amino acid analog incorporation
7. Ease of promoter control
8. Ease of alteration of copy number
9. Ease of formation of intracellular disulfide bonds
10. Ease of growth to very high cell densities
11. Accumulation of heterologous protein up to 50% of dry cell weight
12. Survival under a wide variety of environmental conditions
13. No expensive media requirements
14. Reproducible performance
15. High product yields

Contract (toll) manufacturing is a growing business amounting to $350–450 million per year [25]. In the pipeline are almost 370 products targeting over 200 diseases from biotechnology and pharmaceutical organizations in clinical trials or awaiting FDA approval. An exciting new area in biotechnology is tissue engineering. Skin is already commercially available; cartilage and liver assistance devices are in clinical trails, and the next tissue to be developed is bone.

Production of industrial enzymes was a "natural" for recombinant DNA technology because the products are usually produced from single genes. Over 60% of the enzymes used by the food, detergent and starch-processing industries are recombinant [10]. The market for industrial enzymes rose rapidly in the nineties to reach $2 billion by 2000.

After many years of research showing that random mutation produced many changes in the properties of enzymes, site-directed mutagenesis became a popular technique to improve enzymes. For example, the protease of *Bacillus stearothermophilus* with a heat tolerance of up to 86°C was made resistant to boiling by modifying eight amino acids in the protein structure, all far from the active site of the enzyme [40]. Temperature stability at 100°C increased 340-fold with no loss of stability at 86°C.

Despite the success of site-directed mutagenesis, a much more rapid procedure has recently become very important, i.e., directed evolution of enzymes. Here varying methods are used to randomly make a large number of forms of the enzyme and from such a library, the best is obtained by powerful techniques of selection or screening. The enzyme variants are created by DNA shuffling, error-prone PCR or mutator strains [2,5,28,35,36]. One form of DNA shuffling pools similar genes of different organisms, cuts them with restriction endonucleases and recombines them to form hundreds of new entities. The procedures mimic nature in that they use mutation, selection and recombination to evolve highly adapted proteins but are much faster than nature. Improvements have amounted to 32,000-fold in activity, 1000-fold in substrate specificity, 48-fold in protein folding, 400-fold in antibody activity, 100-fold in antibody expression and 40-fold in inhibitor resistance [30,34].

Of intense current interest is the sequencing of the human genome and those of other organisms. By the end of the 20th century, sequencing had been completed on genomes of 16 bacteria, 6 archaea and 2 eukaryotes. It is anticipated that genome sequencing will result in the discovery of many new pharmaceuticals. The pharmaceutical industry in the future will not only supply drugs but also genes, cells and organelles. It is hoped that this effort will lead to prevention and/or cure of complex diseases such as Alzheimer's disease, Parkinson's disease, cancer, AIDS and many hereditary disorders.

Plant biotechnology has accomplished remarkable feats. Recombinant plants have been produced that are resistant to herbicides, viruses, insects and microbial pathogens. Microbes have played a big part in these scientific developments by providing the Ti plasmid from *Agrobacterium tumefaciens* and the *Bacillus thuringiensis* (Bt) insecticide-encoding gene. In the year 2000, genetically modified soybean reached 54% of total US acreage, cotton reached 68% and corn 25% [1,32]. The total genetically engineered acreage in the US was 70 million acres or 41% of the total; worldwide, it amounted to nearly 100 million acres.

In light of the above triumphs, it is ironic that agricultural biotechnology is bogged down in political controversy in Europe, Asia and the US. [7] This is unfortunate because transgenic plants are absolutely essential in the 21st century. By 2030, the world's population will be 8 billion people (an increase of 2 billion from 2000). Transgenic plants can contribute to feeding this increased population. Such plants promise foods that are more nutritious, stable during storage and health promoting. They contain modified properties such as composition of protein, starch, fats and vitamins by modification of metabolic pathways. They offer increased flexibility in crop management, decreased dependency on chemical pesticides, enhanced yields, easier harvesting and higher proportions of the crop available for trade. Transgenic plants containing the Bt gene have significantly reduced the amount of chemical insecticide needed for cotton. Much coastal and terrestrial landmass has been marginalized due to excess salinity. Salt-tolerance genes from mangroves and *E. coli* have made plants more resistant to salt. By 2000, over 30 million hectares of transgenic plants had been grown with no human health problems by ingestion of such plants or their products. Cereal grains are deficient in iron which causes anemia in pregnant women and children. Four hundred million women suffer and are prone to stillborn or underweight children and mortality at birth in Asia and Africa. This has led to over 20% of maternal deaths after giving birth. Genes encoding an iron-binding protein and an enzyme facilitating iron availability yield transgenic rice with two to four times the usual level of iron [29].

Despite current controversy, Bt corn remains a positive factor to both corn production and insect viability [31]. The negative effects on Monarch and black swallowtail butterfly larvae, feeding on leaves dusted with such pollen, are minimal compared to the positive effect of reduction of pesticide applications. Pesticides are estimated to cause 110,000 nonfatal human poisonings per year in the US and 10,000 cases of cancer and other problems. Thirty-five percent of US supermarket foods have detectable pesticide residues. Some 70 million birds are killed each year by pesticides in the US as well as billions of insects, both harmful and beneficial. Bt corn probably has a net positive effect on the survival of the Monarch butterfly, which is strongly decreased by pesticide exposure.

"Golden Rice" will be very useful to the developing world in which there is severe vitamin deficiency, especially that of vitamin A [15], causing 1–2 million deaths in early childhood and irreversible blindness. "Golden Rice" contains high levels of carotenoids including $\beta$-carotene (provitamin A). The companies developing it have agreed to offer it free of charge to farmers in poor countries. Also available is a high (three-fold) $\beta$-carotene tomato using the bacterial gene *crtI*, which is involved in carotenoid biosynthesis, i.e., converting phytoene to lycopene.

It is clear that the association of industrial microbiology and genetics that began 30 years ago has been more than a casual affair.

## References

1 Anonymous. 2000. Modified soybean, cotton planting up, corn down. *Chem Eng News* 78 (28): 15.
2 Arnold FH. 1998. Design by directed evolution. *Acc Chem Res* 31: 125–131.
3 Bermejo LL, NE Welker and ET Papoutsakis. 1998. Expression of *Clostridium acetobutylicum* ATCC 824 genes in *Escherichia coli* for acetone production and acetate detoxification. *Appl Environ Microbiol* 64: 1079–1085.
4 Bisbee CA. 1993. Current perspectives on manufacturing and scale up of biopharmaceuticals. *Genet Eng News* 13 (8): 8–9.
5 Bornscheuer UT, J Altenbuchner and HH Meyer. 1998. Directed evolution of an esterase for the stereoselective resolution of a key

intermediate in the synthesis of epothilones. *Biotechnol Bioeng* 58: 554–559.
6 Brown K. 1996. Looking back at Jenner, vaccine developers prepare for 21st century. *The Scientist* 10: 14,17.
7 Brown WA. 2000. Facts, beliefs, and genetically modified food. *The Scientist* 14: 39.
8 Chater KF and MJ Bibb. 1997. Regulation of bacterial antibiotic production. In: Rehm HJ, G Reed, H Kleinkauf and H von Döhren (Eds), Biotechnology, Vol. 7, Products of Secondary Metabolism. VCH, Weinheim, pp. 57–105.
9 Cortes J, SF Haydock, GA Roberts, DJ Bevitt and PF Leadlay. 1990. An unusually large multifunctional polypeptide in the erythromycin-producing polyketide synthase of *Saccharopolyspora erythraea*. *Nature* 348: 176–178.
10 Cowan D. 1996. Industrial enzyme technology. *Tibtech* 14: 177–178.
11 Demain AL. 1999. Metabolites, primary and secondary. In: Flickinger MC and SW Drew (Eds), Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation. Wiley, New York, pp. 1713–1732.
12 Donadio S, MJ Staver, JB McAlpine, SJ Swanson and L Katz. 1991. Modular organization of genes required for complex polyketide biosynthesis. *Science* 252: 675–679.
13 Dutton G. 1996. Transgenic animal-based protein products move toward clinical trial. *Genet Eng News* 16 (9): 37.
14 Eggeling L, H Sahm and AA de Graaf. 1996. Quantifying and directing metabolite flux: application to amino acid overproduction. *Adv Biochem Eng* 54: 1–30.
15 Fox S. 2000. Golden Rice intended for developing world. *Genet Eng News* 20 (12): 42,50.
16 Glaser V. 1998. Anti-inflammatory inflames Centocor's profits. *Nat Biotechnol* 16: 900.
17 Hopwood DA, F Malpartida, HM Kieser, H Ikeda, J Duncan, I Fujii, BAM Rudd, HG Floss and S Omura. 1985. Production of "hybrid" antibiotics by genetic engineering. *Nature* 314: 642–644.
18 Hoyle R. 1998. Genentech is poised for an anti-cancer breakthrough. *Nat Biotechnol* 16: 887.
19 Ingram LO, E Conway, DP Clark, GW Sewell and JF Preston. 1987. Genetic engineering of ethanol production in *Escherichia coli*. *Appl Environ Microbiol* 53: 2420–2425.
20 Kennedy J and CR Hutchinson. 1999. Nurturing nature: engineering new antibiotics. *Nat Biotechnol* 17: 538–539.
21 Kerr DE, F Liang, KR Bondioli, H Zhao, G Kreibich, RJ Wall and T-T Sun. 1998. The bladder as a bioreactor: urothelium production and secretion of growth hormone into urine. *Nat Biotechnol* 16: 21,75–78.
22 Khosla C, R Caren, CM Kao, R McDaniel and S-W Wang. 1996. Evolutionally guided enzyme design. *Biotechnol Bioeng* 52: 122–128.
23 Langer LJ. 1999. U.S. biotech symposium focuses on bioprocessing. *Genet Eng News* 19: 1,11,14.
24 Luckow VA and MD Summers. 1988. Trends in the development of baculovirus expression vectors. *Bio/Technology* 6: 47–55.
25 McCoy M. 1999. Setting course for prosperity. *Chem Eng News* 77: 29,30,32–34.
26 McKown RL and RA Teutonico. 1999. Transgenic animals for production of proteins. *Genet Eng News* 19 (9): 1,14,29,46,55,60.
27 Miller LK. 1988. Baculoviruses as gene expression vectors. *Annu Rev Microbiol* 42: 177–199.
28 Minshull J and WPC Stemmer. 1999. Protein evolution by molecular breeding. *Curr Opin Biotechnol* 3: 284–290.
29 National Academy of Sciences USA. 2000. Transgenic Plants and World Agriculture. National Academy Press, Washington, DC.
30 Patten PA, RJ Howard and WPC Stemmer. 1997. Applications of DNA shuffling to pharmaceuticals and vaccines. *Curr Opin Biotechnol* 8: 724–733.
31 Pimentel DS and PH Raven. 2000. Bt corn pollen impacts on nontarget lepidoptera: assessment of effects in nature. *Proc Natl Acad Sci USA* 97: 8198–8199.
32 Prakash CS. 2000. Hungry for biotech. *Technol Rev* 103: 32.
33 Riesenberg D and R Guthke. 1999. High-cell-density cultivation of microorganisms. *Appl Microbiol Biotechnol* 51: 422–430.
34 Skandalis A, LP Encell and LA Loeb. 1997. Creating novel enzymes by applied molecular evolution. *Chem Biol* 4: 889–898.
35 Spee JH, WM de Vos, OP Kuipers. 1993. Efficient random mutagenesis method with adjustable mutation frequency by use of PCR and dITP. *Nucleic Acids Res* 21: 777–778.
36 Stemmer WPC. 1994. DNA shuffling by random fragmentation and reassembly: *in vitro* recombination for molecular evolution. *Proc Natl Acad Sci USA* 91: 10747–10751.
37 Swartz JR. 1996. *Escherichia coli* recombinant DNA technology. In: Neidhardt FC (Ed), *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology. American Society of Microbiology (ASM) Press, Washington, DC, pp. 1693–1711.
38 Thayer A. 1996. Market, investor attitudes challenge developers of biopharmaceuticals. *Chem Eng News* 74 (33): 13–21.
39 Thayer A. 2000. Biotech industry boosts US economy. *Chem Eng News* 78 (31): 26.
40 Van den Burg B, G Vriend, OR Veltman, G Venema and VGH Eusink. 1998. Engineering an enzyme to resist boiling. *Proc Natl Acad Sci USA* 95: 2056–2060.