0273-2289/93/3801-02—0105$08.20

Cellular and Metabolic Engineering

An Overview

DOUGLAS C. CAMERON* AND I-TEH TONG

Department of Chemical Engineering,
University of Wisconsin-Madison,
1415 Johnson Drive, Madison, WI 53706-1691

ABSTRACT

Metabolic engineering is defined as the purposeful modification of intermediary metabolism using recombinant DNA techniques. Cellular engineering, a more inclusive term, is defined as the purposeful modification of cell properties using the same techniques. Examples of cellular and metabolic engineering are divided into five categories:

1. Improved production of chemicals already produced by the host organism;
2. Extended substrate range for growth and product formation;
3. Addition of new catabolic activities for degradation of toxic chemicals;
4. Production of chemicals new to the host organism; and
5. Modification of cell properties.

Over 100 examples of cellular and metabolic engineering are summarized. Several molecular biological, analytical chemistry, and mathematical and computational tools of relevance to cellular and metabolic engineering are reviewed. The importance of host selection and gene selection is emphasized. Finally, some future directions and emerging areas are presented.

Index Entries: Metabolic engineering; cellular engineering; strain improvement.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Cellular engineering and metabolic engineering are interrelated fields of broad practical and basic importance, made possible by the development of recombinant DNA technology. Both fields are highly multidisciplinary, drawing on information and techniques from biochemistry, genetics, molecular biology, cell physiology, chemistry, chemical engineering, and systems analysis.

Although cellular engineering, metabolic engineering, and related terms are used widely in the scientific and technical literature, there are currently no clear-cut definitions of these terms. Some of the more commonly used terms and their definitions are listed in Table 1. Metabolic engineering is most often used for applications in industrial microbiology and bioprocess engineering. The terms in vitro evolution and directed evolution are used to describe both the directed modification of organisms using recombinant DNA technology and the use of strong selective pressures, such as can be obtained in a chemostat. The terms are primarily used in the context of environmental microbiology. Molecular breeding is synonymous with metabolic engineering, and is the favored term of Japanese researchers. Cellular engineering is generally used to describe work involving the modification of animal and plant cells, but is also used for bacteria, and is the most inclusive of the terms. Other terms, such as plasmid-assisted molecular breeding (*6*) and plasmid-based catalysis (*9,10*), are overly restrictive in that much of the current work in cell modification involves the integration of genes into the chromosome. A final, although less glamorous, description of much of the work reviewed here is "rational strain development."

For this article, metabolic engineering is defined as the purposeful modification of intermediary metabolism using recombinant DNA techniques. Cellular engineering is defined as the purposeful modification of cell properties using such techniques. Cellular engineering encompasses much of what is called metabolic engineering. However, it is not all-inclusive, since metabolic pathways can be modified and used apart from the cell or in artificial cells (e.g., in vitro multienzyme systems).

This article is divided into four parts. The first is a summary of examples of cellular and metabolic engineering. The purpose of this section is primarily to demonstrate the scope of these areas. A detailed literature search of almost any of the examples described in this section will reveal numerous related papers and patents. In addition, the number of examples of cellular and metabolic engineering is increasing rapidly. The second part is a review of various tools of use to the cellular or metabolic engineer. The third part is a discussion of guidelines and general considerations for pathway and cell modification. The final part is a discussion of future directions. Additional aspects of cellular and metabolic engineering have recently been reviewed by Nerem (*8*) and Bailey (*1*).

Table 1
Definitions and Terms Related to Metabolic Engineering

Term	Definition	Reference
Metabolic engineering	"Improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology"	Bailey, 1991 (*1*)
(Microbial) pathway engineering	"Modification of various metabolic pathways using recombinant techniques so as to enhance production of a particular metabolite or indeed to include pathways not indigenous to the organism"	MacQuitty, 1988 (*2*)
Metabolic pathway engineering	"The modification, design, and construction of biochemical pathways"	Tong et al., 1991 (*3*)
In vitro evolution	"...cloned and well characterized genes are selectively transferred into a different organism in order to evolve a new pathway"	Timmis et al., 1988 (*4*)
Directed evolution	"The recruitment of genes in a stressed environment"	Chakrabarty, 1992 (*5*)
Molecular breeding	"Breeding" of new strains of organisms via direct manipulation the genes/DNA at the molecular level	Kellogg et al., 1981 (*6*) Kanayama et al., 1988 (*7*)
Cellular engineering	"Application of the principles and methods of engineering to problems in cell and molecular biology of both a basic and applied nature"	Nerem, 1991 (*8*)

EXAMPLES OF CELLULAR AND METABOLIC ENGINEERING

There are numerous applications of cellular and metabolic engineering described in the scientific and patent literature. In this article, examples of these applications are divided into the following categories:

1. Improved production of chemicals already produced by the host organism;
2. Extended substrate range for growth and product formation;
3. Addition of new catabolic activities for degradation of toxic chemicals;
4. Production of chemicals new to the host organism; and
5. Modification of cell properties.

The categories are somewhat arbitrary, and some examples fit into more than one category; e.g., the work of Wood and Ingram (*11*) involves both improving ethanol production by *Klebsiella oxytoca* and extending the substrate range of the organism. In order to keep this article to a reasonable size, only examples that demonstrate product formation or some clearly altered cell property are presented. The scope of the article has been further restricted by not considering the modification of cells for the production of proteins or examples involving protoplast fusion.

Improved Production of Chemicals Already Produced by the Host Organism

Examples of metabolic engineering to improve chemical production are listed in Table 2. The host organism refers to the organism (cell) that is the recipient of the new genetic material or that has its genetic make-up altered. The new genetic material can be from the host itself or from a foreign organism. The examples are divided into catabolic end products, antibiotics, vitamins and amino acids, polymers, and other chemicals.

In the area of catabolic end products, by far the most work has been done on ethanol production. The primary host organisms of interest have been *Escherichia coli* and *Klebsiella* species, primarily because of the wide substrate range of these organisms and the powerful molecular biological tools available for their manipulation. Both of these organisms naturally ferment sugars to small amounts of ethanol, but with the addition of pyruvate decarboxylase (PDC) from *Zymomonas mobilis* or both PDC and alcohol dehydrogenase from *Z. mobilis*, they are excellent ethanol producers, and can utilize glucose, xylose, and lactose. In addition to the work described in Table 2, much work has been done on the characterization and analysis of metabolically engineered ethanol producers (*53–58*). Another active area is butanol production by *Clostridium acetobutylicum* (*26,59*); however,

since *C. acetobutylicum* is a more difficult organism to work with than *E. coli*, and much less is known about its genetics and physiology, the progress in metabolic engineering has been fairly slow. Once the technical barriers are overcome, metabolic engineering of butanol production is likely to develop rapidly.

Metabolic engineering of antibiotic production is a huge area of great practical importance and is just briefly touched on in this article. The work has focused on improving the production of known antibiotics, the modification of known antibiotics to improve their properties, and the discovery of new antibiotics (*31*). Some examples of improving the production of antibiotics by their natural producer are summarized in Table 2. (Examples of the production of modified or new antibiotics and of the production of known antibiotics in an new host are summarized in Table 3). Two factors that make antibiotic production particularly attractive for metabolic engineering are that antibiotic genes are generally clustered and, thus, easier to clone than might be initially expected, and that the genes are often positively regulated, so it is possible to improve production by overexpressing the regulator (*97*).

One of the most impressive examples of metabolic engineering is the improvement of phenylalanine production. Phenylalanine is of commercial importance for the production of the sweetener aspartame. For example, Backman et al. (*42*) have cloned and expressed multiple genes in the host organism, altered the regulatory properties of the organism at the genetic and enzymatic levels, and used a variety of special techniques, such as excision vectors, to improve production. The engineered strain produced over 50 g/L of phenylalanine.

The improvement of polymer production by organisms is of great practical importance. The two examples listed in Table 2 are xanthan gum and bacterial cellulose. Another polymer of interest is polyhydroxybutyrate (PHB) (*66*). However, the greatest potential in this area is the production of novel polymers and polymers that are new to the host organism (Table 3).

Metabolic engineering can be used to alter organisms so that they will overproduce practically any metabolic intermediate. Several compounds that have been overproduced using metabolic engineering techniques include peptides, lipids, and intermediates of aromatic amino acid synthesis (Table 2). Clearly, the range of potential products is enormous.

Extended Substrate Range for Growth and Product Formation

A very practical aspect of metabolic engineering is the extension of the substrate range that a host organism can use for growth and product formation. Several examples of this work are given in Table 4. Most of the work has focused on engineering organisms to use lactose, a major

Table 2
Improved Production of Chemicals (Other than Proteins) Normally Produced by the Host Organism

Chemical	Host organism	Notes	Reference
Catabolic end products			
CO_2	*Saccharomyces cerevisiae*	Added fructose-1,6-diphosphatase gene to create a futile cycle to enhance CO_2 production	Rogers and Szostak, 1987 (*12*)
H_2	*E. coli*	Expressed hydrogenase gene from *Citrobacter freudii*	Kanayama et al., 1988 (*7*)
Acetic acid	*Acetobacter aceti*	Expressed membrane-bound aldehyde dehydrogenase gene from *Acetobacter polyoxogenes*, resulting in increased production rate and acetic acid concentration	Fukaya et al., 1989 (*13*)
Ethanol	*E. coli*	Cloned and expressed pyruvate decarboxylase from *Zymomonas mobilis*	Brau and Sahm, 1986 (*14*)
Ethanol	*Erwinia chrysanthemi*	Expressed pyruvate decarboxylase from *Zymomonas mobilis;* increased ethanol yields from xylose and arabinose, but decreased growth rate and ethanol tolerance	Tolan and Finn, 1987 (*15*)
Ethanol	*Klebsiella planticola*	Expressed pyruvate decarboxylase from *Zymomonas mobilis;* increased ethanol yield from xylose	Tolan and Finn, 1987 (*16*)
Ethanol	*E. coli*	Expressed pyruvate decarboxylase from *Zymomonas mobilis* in mutant *E. coli* with hyperexpressing alcohol dehydrogenase	Ingram and Clark, 1991 (*17*)
Ethanol	*E. coli*	Constructed *pet* operon consisting of pyruvate decarboxylase and alcohol dehydrogenase II genes from *Zymomonas mobilis;* efficient ethanol production from glucose, xylose, and lactose	Ingram et al., 1987 (*18*); Ingram and Conway, 1988 (*19*); Alterthum and Ingram, 1989 (*20*); Ingram et al., 1990 (*20*); Ingram et al., 1991 (*22*)

Ethanol	*E. coli*	Pyruvate decarboxylase and alcohol dehydrogenase II genes from *Zymomonas mobilis* were integrated into *E. coli* chromosome; improved stability of genes over plasmid-based system	Ohta et al., 1991 (*23*)
Ethanol	*Klebsiella oxytoca*	Expressed pyruvate decarboxylase and alcohol dehydrogenase genes from *Zymomonas mobilis;* ethanol productivity greater than with *E. coli* from glucose and xylose	Ohta et al., 1991 (*24*)
Ethanol	*Klebsiella planticola*	Expressed pyruvate decarboxylase gene from *Zymomonas mobilis* in a pyruvate-formate-lyase mutant; 41 g/L of ethanol from xylose	Feldmann et al., 1989 (*25*)
Butanol, acetone	*Clostridium acetobutylicum*	Expressed acetoacetate decarboxylase and phosphotransbutyrylase from *C. acetobutylicum* using a *Bacillus subtilis/C. acetobutylicum* shuttle vector; no information on product information	Mermelstein et al., 1992 (*26*)
Antibiotics			
Tylosin	*Streptomyces fradiae*	Expressed *tyl*F gene from *E. coli,* resulting in 14- to 18-fold increase in tylosin production	Cox et al., 1987 (*27*)
Cephamycin C	*Streptomyces lactamgens*	Expressed genes from *S. cattleya* (genes also enabled product formation in a previously nonproducing host)	Chen et al., 1988 (*28*)
Cephalosporin C	*Cephalosporium acremonium*	Expressed *def*EF genes in an industrial strain, resulting in higher productivity	Skatrud et al., 1989 (*29*)
LL-E33288 complex (antitumor and antibacterial agent)	*Micromonospora echinospora*	Overexpressed *M. echinospora* genes to get higher yields; also produced altered products with lower toxicity	Rothstein et al., 1991 (*30*)

Table 2 (*continued*)

Doxorubicin	*Streptomyces peucetius*	Extra copies of genes resulted in a 10-fold overproduction of daunorubicin, the penultimate precursor of doxorubicin	Hutchinson, 1992 (*31*)
Spiramycin and other macrolide antibiotics	*Streptomyces ambofaciens*	Increased level of spiramycin; also hybrid antibiotics	Beckmann et al., 1992 (*32*)
Vitamins and amino acids			
Biotin	*E. coli*	Cloned five biotin synthesis genes; all five genes made free from feedstock inhibition	Ifuku et al., 1986 (*33*)
L-lysine	*Corynebacterium* and *Brevibacterium*	Amplified dihydrodipicolinate synthetase and tetrahydropicolinic acid succinylase; 20 g/L lysine	Katsumata et al., 1986 (*34*)
L-asparate	*Serratia marcescens*	Overexpressed the aspartase gene from *S. marcescens;* organism converts fumarate and ammonia to aspartate	Kisumi and Takagi, 1987 (*35*)
L-threonine	*Serratia marcescens*	Amplified phosphoenolpyruvate carboxylase (*ppc*) genes from *E. coli;* 63 g/L of threonine	Sugita and Komatsubara, 1989 (*36*)
L-arginine	*Corynebacterium* and *Brevibacterium*	Cloned arginine biosynthetic genes *Corynebacterium acetoacidophilum;* 3.2 g/L of arginine	Katsumata and Yokoi, 1988 (*37*)
L-glutamic acid and L-proline	*Corynebacterium* and *Brevibacterium*	Expressed the citric acid synthetase from *E. coli;* 31.2 g/L of glutamic acid	Katsumata et al., 1988 (*38*)
L-glutamic acid	*Corynebacterium* and *Brevibacterium*	Expressed the phosphofructokinase gene; 32.6 g/L glutamic acid	Katsumata et al., 1988 (*39*)

L-phenylalanine	*E. coli*	Expressed all phenylalanine genes except transaminases from *E. coli;* deletion of repressor protein; engineered new promotor for *phe*A; genetic alteration to overcome feedback inhibition of chorismate mutase; excision vector for tyrosine biosynthetic gene; selected for resistance to toxic amino acid analogs; growth and production at 37°C; >50 g/L of product	Backman and Balakrishnan, 1988 (*40*); Backman, 1989 (*41*); Backman et al., 1990 (*42*)
L-phenylalanine	*E. coli*	Expressed *phe*A and *aro*F genes from *E. coli* under control of a temperature sensitive promoter from phage λ; optimal product formation at 30–32°C; 43.7 g/L of product	Lee et al., 1991 (*43*)
L-phenylalanine, L-tyrosine	*Corynebacterium glutamicum*	Overexpressed prephenate dehydratase, chorismate mutase (CM) and 3-D-*arabino*-heptulosonate synthase (DS) to direct flux to phenylalanine; overexpressed CM and DS to direct flux to tyrosine; metabolic regulation is a key factor	Ikeda and Katsumata, 1992 (*44*)
Polymers			
Xanthan gum	*Xanthomonas campestris*	Overexpressed xanthan synthesis genes, resulting in higher productivity and final gum concentration	Pollock and Thorne, 1988 (*45*)
Bacterial cellulose	*Acetobacter xylinum*	Cloned and expression cellulose biosynthesis operon of *A. xylinlum;* demonstrated increased cellulose synthesis	Ben-Bassat et al., 1990 (*46*); Wong et al., 1990 (*47*)

Table 2 (*continued*)

Other chemicals			
S-D-lactoylglutathione	*E. coli*	Review article; overexpressed glyoxalase I from *Pseudomonas putida*	Murata and Kimura, 1990 (*48*)
glutathione and derivatives	*E. coli*	Review article; overexpressed γ-L-glutamyl-L-cysteine synthase and glutathione synthetase from *E. coli*	
S-adenosyl-L-methionine (SAM)	*Saccharomyces cerevisiae* preferred, also *E. coli*	Enhanced intracellular accumulation of SAM by expression of "SAM gene"; 40 mg/g-dry cell of SAM	Shiomi and Fukuda, 1992 (*49*)
3-deoxy-D-*arabino*-heptulosonates (DAH and DAHP)	*E. coli*	Increased production of intermediates in aromatic amino acid biosynthesis by overexpression of *E. coli* transketolase and DAHP synthase	Frost et al., 1990 (*50*); Draths and Frost, 1990 (*10*); Draths et al., 1992 (*51*)
3-Dehydroshikimate (DHS)	*E. coli*	Increased production of DHS, an intermediate in aromatic amino acid biosynthesis by overexpression of *E. coli* transketolase, DAHP synthase and DHQ synthase in an *E. coli* mutant	Draths and Frost, 1990 (*9*); Draths et al., 1992 (*51*)
Lipids	Microalgae	Preliminary work on engineering microalgae species for fuel production	Dunahay et al. 1992 (*52*)

byproduct of the cheese-making industry, and xylose, the primary five-carbon sugar in biomass. Work has also focused on the utilization of cellulose. For complex substrates, such as cellulose, the approach that has been most often used is to clone hydrolytic enzymes into the cell. However, a difficulty with this approach is that the enzymes must be secreted into the medium to have access to the substrate. Wood and Ingram (*11*) have gotten around this problem for ethanol production from cellulose by developing a multistage process that involves lysing the cells to release the enzyme. In the process, a temperature-stable cellulase gene from *Clostridium thermocellum* cloned on a plasmid is added to *Klebsiella oxytoca*, which has the *Zymomonas mobilis* ethanol production genes stably integrated into the chromosome. In order to start up the process, the cells are grown on a readily usable substrate, such as glucose. The cells from the first batch are then lysed, and the cellulase is used to hydrolyze cellulose. The medium is then inoculated with the same type of engineered cells, and the process is repeated.

For simple sugars, such as lactose, the engineered cells need both the proper sugar transport system and the necessary catabolic enzymes. The importance of transport systems in metabolic engineering is discussed by Romano (*106*). Lactose utilization is a relatively easy property to engineer in an organism because much is known about the lactose operon. The addition of xylose-utilizing ability to the yeast *Saccharomyces cerevisiae* is of great interest, but has proven to be difficult (*107*). Much early work focused on engineering xylose isomerase in yeast. Another potential approach to xylose utilization in *S. cerevisiae* is to add genes for enzymes that catalyze the reduction of xylose to xylitol and the oxidation of xylitol to xylulose (*108*).

Addition of New Catabolic Activities for the Degradation of Toxic Chemicals

The first example of metabolic engineering (in 1974) was the construction of several *Pseudomonas* strains able to catabolize a wide range of substrates, including camphor and naphthalene (*109*). Some further examples of the addition of new catabolic activities for the degradation of toxic chemicals are given in Table 5. Both the recombinant DNA approach (which in the case of adding new catabolic activities is often called in vitro [or laboratory] evolution [*4*]) and other less precise approaches (such as intergenetic mating [*114*]) are used. Work in this area is difficult, partly because of the lack of knowledge of the relevant biochemical pathways and partly because of the unfavorable energetics of the utilization of some substrates, problems with transport of sparingly soluble substrates, the generally low substrate concentrations, and substrate toxicity (especially when the substrate concentration is high).

Table 3
Production of Chemicals (Other than Proteins) New to the Host Organism

Chemical	Host organism	Notes	Reference
Antibiotics			
Mederrhodin and dihydro-granatirhodin	*Streptomyces sp.* and *Streptomyces violaceoruber*	Added biosynthetic gene cluster from *Streptomyces coelicolor*, resulting in production of two hybrid antibiotics	Hopwood et al., 1985 (*60*)
Erythromycin	*Streptomyces lividans*	Expressed *Streptomyces erythreus* genes; low levels of production	Stanzak et al., 1986 (*61*)
Modified erythromycin	*Saccharopolyspora erythraea*	6-Deoxyerythromycin A, a derivative of erthythromycin with increased acid stability, produced by organism with targeted disruption in *eryF* gene	Weber et al., 1991 (*62*)
Isovaleryl-spiramycin	*Streptomyces ambofaciens*	Added genes from *Streptomyces thermotolerans*, resulting in the formation of new antibiotics	Epp et al., 1989 (*63*)
Penicillin V	*Neurospora crassa* and *Aspergillus niger*	Added native penicillin biosynthetic gene cluster from *Penicillium chrysogenum*	Smith et al., 1990 (*64*)
Spiramycin/hybrid macrolides	*Streptomyces ambofaciens*	Use of mutant spiromycin biosynthetic genes	Beckmann et al., 1992 (32)
Polymers			
Polyhydroxy butyrate (PHB)	*E. coli*	Added PHB biosynthetic genes from *Alcaligenes eutrophus*, resulting in the synthesis of PHB	Slater et al., 1988 (65); Peoples and Sinskey, 1990 (*66*)
Polyhydroxy alkanoates (PHAs)	*Pseudomonas oleovoran*	Added PHB biosynthetic genes from *Alcaligenes eutrophus*, resulting in the synthesis of PHA copolymers	Timm et al., 1990 (*67*)

Poly-(3-hydroxy-butyrate-co-3-hydroxyvalerate)	*E. coli*	Expressed the PHB operon from *Alcaligenes eutrophus* in a strain of *E. coli* with constitutive expression of acetate and propionate utilization; substrates were glucose and propionate	Slater et al., 1992 (*68*)
Polyhydroxbutyrate (PHB)	*Arabidopsis thaliana*	Expressed acetoacetyl-CoA reductase and PHB synthase from *Alcaligenes eutrophus* in a plant	Poirier et al., 1992 (*69*)
Modified xanthan gums	*Xanthomonas campestris*	Made specific mutations to modify five sugar repeating unit of xanthan gum	Hassler and Doherty, 1990 (*70*)
Melanin	*E. coli*	Added tyrosinase genes from *Strepomyces antibioticus*	della-Cioppa et al., 1990 (*71*)
Other chemicals			
Indigo	*E. coli*	Expressed "indigo operon" containing the naphthalene dioxygenase gene from *Pseudomonas putida* and tryptophanase gene; cells able to convert tryptophan to cis-indole-2,3-dihydrodiol, which rearranges to form indoxyl and, in the presence of air, is converted to indigo	Ensley et al., 1983 (*72*) Ensley et al., 1985 (*73*)
Indigo	*E. coli*	Expressed *Pseudomonas putida* gene for xylene oxidase; conversion of indole directly to indoxyl (3-hydroxyindole) followed by nonenzymatic conversion to indigo in the presence of air	Mermod et al., 1986 (*74*); Keil et al., 1987 (*75*)
Indigo (and other pigments)	*E. coli*	Expressed *Pseudomonas putida* toluene dioxygenase gene (and other genes), resulting in production of blue-green pigment with indigo a major component	Stephens et al., 1989 (*76*)

Table 3 (*continued*)

Indigo	*E. coli*	Expressed *Rhodococcus* indole dioxygenase gene	Hart and Woods, 1992 (*77*); Hart et al., 1992 (*78*)
2-keto-L-gluconic acid (2-KLG)	*Erwinia herbicola*	Added to the 2,5-diketo-D-gluconate (2,5-DKG) reductase gene from *Corynebacterium* sp. ATCC 31090, resulting in the direct synthesis of 2-KLG (a precursor of vitamin C) from glucose	Anderson et al., 1985 (*79*); Lazarus et al., 1990 (*80*); Anderson et al., 1991 (*81*)
2-keto-L-gluconic acid (2-KLG)	*Erwinia citreus*	Similar to above, but used 2,5-DKG reductase gene from *Corynebacterium* sp. SHS 752001	Hardy et al., 1990 (*82*)
Acetoin	*E. coli*	Added α-acetolactate decarboxylase gene from *Streptococcus lactis*, resulting in the production of acetoin	Goelling and Stahl, 1988 (*83*)
L-tyrosine	*Corynebacterium* and *Brevibacterium*	Added 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase and chorismate mutase genes, resulting in the accumulation of 8 g/L of tyrosine	Katsumata and Ikeda, 1988 (*84*)
Quinolinic acid	*E. coli*	Added *nadA* and *nadB* genes from *E. coli*, resulting in the production of 2.32 g/L of quinolinic acid	Laeufer et al., 1988 (*85*)
1,3-Propanediol (1,3-PD)	*E. coli*	Expressed glycerol dehydratase and 1,3-PD oxidoreductase from *Klebsiella pneumoniae dha* regulon for production of 1,3-PD from glycerol; enhanced 1,3-PD production by cofermentation of glycerol and sugars	Tong et al., 1991 (3); Tong and Cameron, 1992 (*86*) Tong, 1992 (*87*)
Catechol	*E. coli*	Expressed transketolase, DAHP synthase, and DHQ synthase in various *E. coli* mutants, thereby inadvertently inducing a pathway for catechol production	Draths and Frost, 1991 (*88*)

Xylitol	*Saccharomyces cerevisiae*	Added xylose reductase gene from *Pichia stipitis*, resulting in xylitol production from xylose	Hallborn et al., 1991 (*89*)
Octanoic acid	*E. coli*	Added *alk* genes from the *Pseudomonas oleovoran* OCT plasmid, resulting in the bioconversion of N-octane to octanic acid	Favre-Bulle et al., 1991 (*90*)
Cyclodextrins	Potato plants	Added cyclodextrin glycosyltransferase gene from *Klebsiella pneumoniae*, resulting in cyclodextrin synthesis in tubers of transgenic potatoes	Oakes et al., 1991 (*91*)
Alkaloids	*Atropa belladonna, Nicotiana tabacum,* and *Solanum tuberosum*	Introduced *Agrobacterium* Ti and Ri plasmids into the host plants, resulting in the accumulation of alkaloids	Saito et al., 1991 (*92*)
β-Carotene	*Zymomonas mobilis* and *Agrobacterium tumefaciens*	Expressed *crtB, crtI, crtY* genes from *Erwinia uredovra*, resulting in the production of β-carotene	Misawa et al., 1991 (*93*)
geranylgeranyl pyrophosphate, phytoene, lycopene, β-carotene, xeaxanthin, and zeaxanthin diglucoside	*Saccharomyces cerevisiae*	Cloned six carotenoid pathways genes from *Erwinia herbicola;* different products produced depending on the number of genes in the linear pathway that are expressed	Ausich et al., 1991 (*94*)
Laurate	*Arabidopsis thaliana*	Expressed key enzyme in medium-chain fatty acid synthesis (12:0-acyl-carrier protein thioesterase) from California bay	Voelker et al., 1992 (*95*)
Long-chain dicarboxylic acids	*Candida tropicalis*	Disrupted four genes for the isozymes of acyl-CoA oxidase used in β-oxidation in order to redirect alkane and fatty acid substrates to ω-oxidation pathway; amplfied P450 monooxygenase and the NADPH-cytochrome reductase genes to improve productivity	Picataggio et al., 1992 (*96*)

Table 4
Extension of Substrate Range for Growth and Product Formation

Substrate	Host organism	Notes	Reference
Lactose	*Leuconostoc cremoris*	Transferred lactose-fermenting ability from *Streptococcus lactis;* conjugants also produced diacetyl	Tsai and Sandine, 1987 (*98*)
Lactose	*Pseudomonas aeroginosa*	Integrated *E. coli lac*YZ into *P. aeruginosa* chromosome; recombinant cells produced rhamnolipid biosurfactants from lactose	Koch et al., 1988 (*99*)
Whey/lactose	*Xanthomonas campestris*	Expressed *lac*YZ genes from *E. coli* with *lpp* ribosome binding site; xanthan gum production from whey	Fu and Tseng, 1990 (*100*)
Lactose and galactose	*Alcaligenes eutrophus*	Added *lac* and *gal* operons from *E. coli;* polyhydroxyalkanoate production from lactose and galactose	Pries et al., 1990 (101)
Lactose, mannitol, and others	*Zymomonas mobilis*	Review of work on extending substrate range for ethanol production	Buchholz and Eveleigh, 1990 (*102*)

Whey/lactose	*Saccharomyces cerevisiae*	Expressed *Aspergillus niger* β-galactosidase gene; growth on whey	Kumar et al., 1992 (*103*)
Xylan	*E. coli; Klebsiella oxytoca*	Expressed xylanase gene from *Clostridium thermocellum* in strains previously engineered for ethanol production	Burchhardt and Ingram, 1992 (*104*)
Cellobiose, amorphous cellulose, crystalline cellulose	*Klebsiella oxytoca*	Expressed endoglucanase from *Clostridium thermocellum* in a strain previously engineered for ethanol production; process involves growth of cells on an easily used substrate, cell lysis to release enzymes, hydrolysis of cellulose, and then fermentation of hydrolyzed products to ethanol	Wood and Ingram, 1992 (*11*)
Sucrose	*E. coli* K12	Expressed sucrose utilization system from *E. coli* B-62 in *E. coli* K12; demonstrated tryptophan production from sucrose	Tsunekawa et al., 1992 (*105*)

Table 5
Addition of New Catabolic Activities for Detoxification, Degradation, or Mineralization of Toxic Chemicals

Chemical	Host organism	Notes	Reference
Camphor, salicylatic acid, naphthalene	*Pseudomonas putida, Pseudomonas aeruginosa*	Added two or more stable plasmids specifying separate degradative pathways	Chakrabarty, 1974 (*109*)
Chloro- and methyl-aromatics	*Pseudomonas* sp.	Critical enzymes from five different aromatic catabolic pathways of three different soil bacteria combined into a functional ortho cleavage route for the degradation of methylphenols and methylbenzoates	Rojo et al., 1987 (*110*)
4-Ethylbenzoate	*Pseudomonas putida*	Added genes from mutant bacteria selected for their production of altered regulator or altered catechol 2,3-dioxygenase with lower sensitivity to metabolite inactivation	Ramos et al., 1987 (*111*)
Trichloroethane	*E. coli*	Added genes from *Pseudomonas mendocina* to degrade trichloroethane	Winter et al., 1989 (*112*)
Polychlorinated biphenyls (PCBs)	*E. coli*	Added genes from *Pseudomonas* sp. to degrade PCBs	Mondello, 1989 (*113*)
3-Chlorobiphenyl	*Pseudomonas* sp.; *Acinetobacter* sp.	Intergeneric mating of a *Pseudomonas* strain able to grow on 3-chloro-benzoate with *Acinetobacter* strain able to grow on biphenyl in a continuous amalgamated culture apparatus	Adams et al., 1992 (*114*)

Production of Chemicals New to the Host Organism

This is one of the most exciting applications of metabolic engineering. Several examples of the production of chemicals new to the host organism are given in Table 3. The table is divided into sections on antibiotics, polymers, and other chemicals. The area of greatest activity has been the production of antibiotics, with the primary focus on the discovery of new (hybrid) antibiotics. It is beyond the scope of this article to discuss the vast amount of research on the discovery of new antibiotics (for reviews, *see 31,97,115,116*). The finding that many antibiotics genes are clustered and that some genes of related pathways show crosshybridization has made such work somewhat easier (*97*). One consideration with hybrid antibiotics is that the host organisms must be resistant to the antibiotic in order to give good production.

The production of new polymers is another major application of metabolic engineering. Peoples and Sinskey (*66*) have called this area biopolymer engineering. One large area of biopolymer engineering, the use of recombinant DNA technology for the production of protein-based materials, is not reviewed here. The genes for the polyhydroxybutyrate (PHB) pathway in *Alcaligenes eutrophus* have been cloned by several research groups, and there is a major worldwide thrust to develop metabolically engineered cells to produce PHB and derivatives of this biodegradable plastic. Recently, this pathway was cloned and expressed in a plant, although very little PHB was produced (*69*). Another major area of opportunity is the genetic modification of polysaccharides. One example is the genetic modification of xanthan gum (*70*). Another example is the cloning of the xanthan gum biosynthetic pathway into an organism capable of anaerobic respiration (Dohery, unpublished). One of the major costs in polysaccharide production is the energy required for aeration. Organisms capable of anaerobic respiration can use very soluble compounds, such as nitrate, instead of oxygen (which has low water solubility), as the terminal electron acceptor. Presumably the savings in aeration costs will make up for the lower metabolic efficiency of anaerobic vs aerobic respiration.

Two "classical" examples of metabolic engineering are the production of indigo and the vitamin C precursor, 2-keto-L-gluconic acid (2-KLG). The production of indigo by *E. coli* containing a genetically engineered naphthalene dioxygenase gene was a serendipitous discovery (*72,73*). Indigo may be one of the first commercial products of a metabolically engineered organism (*117*). Since these original developments, alternate metabolic engineering approaches for both 2-KLG (*82*) and indigo (*74–78*) have been reported.

A growing number of other chemicals have been produced by metabolically engineered organisms. Draths and Frost (*88*) reported the production of catechol in an engineered strain of *E. coli* (the strain was not

engineered to produce catechol, but the presence of the engineered genes evidently caused the induction of silent genes or, in some other way, redirected the metabolic flux). Tong et al. (*3*) recently constructed a metabolic pathway for 1,3-propanediol (1,3-PD) in *E. coli*. The pathway was constructed as a model system for the investigation of metabolic engineering and also as the initial step in the development of a process for the production of 1,3-PD, a polymer intermediate, from glycerol and other renewable resources.

Modification of Cell Properties

Examples of the modification of cell properties are given in Table 6. Some of these are not examples of metabolic engineering because they do not involve the manipulation of intermediary metabolism; they are, however, examples of cellular engineering as defined in the Introduction. The examples involve a wide range of organisms, from bacteria to animal cells, and include a diverse set of properties, from improved growth rate to glucose-stimulated insulin secretion.

The replacement of the energetically less efficient GOGAT ammonia uptake system with the GDS in *Methylophilis methylotrophus* (*119*) in order to improve the efficiency of the large-scale production of bacterial protein is one of the first industrial examples of cellular engineering (and perhaps the first large-scale use of a genetically engineered microorganism). This work exemplifies the principle that properties of organisms that are necessary for survival in nature are not necessarily desirable in the controlled environment of a bioreactor.

Another significant development in cellular engineering is the expression of the *Vitreoscilla* hemoglobin gene in bacteria (*120,122*) and other organisms, to improve growth and product formation under oxygen-limiting conditions common in industrial bioreactors with high cell densities. The expression of the hemoglobin gene has potential commercial applications in processes for the production of proteins, antibiotics, and amino acids (*142*).

Another class of examples involves the improvement of the "hardiness" of the cell or organism. Various genes for salt tolerance, drought tolerance, pathogen resistance, and resistance to toxic metabolic intermediates have been identified and expressed in cells. Two related areas of great practical importance that are not included in Table 6 are antibiotic resistance and phage resistance.

Cells have recently been engineered for use in bioassays of toxic chemicals. Buchholz et al. (*131*) have developed a cell line that can be used for the detection of promutagenic chemicals. Previous systems have required the use of liver homogenates. Rosson has developed a whole-cell bioluminescence-based system for detecting toxic heavy metals (*130*).

Table 6
Modification of Cell Properties

Property	Host organism	Notes	Reference
Improved growth yield	*Methylophilus methylotrophus*	Achieved more efficient ammonia uptake system by replacing glutamate synthetase/glutamine synthetase (GOGAT) system with glutamine dehydrogenase (GDH) system	Windass et al., 1980 (*118*); Senior and Windass, 1980 (*119*)
Increased cell density	*E. coli*	Expressed ethanol production genes from *Zymomonas mobilis* to redirect flux from acetate to ethanol, a less toxic intermediate	Ingram et al., 1987 (*18*); Ingram and Conway, 1988 (*19*); Ingram et al., 1991 (*22*)
Enhanced growth and other oxygen-related processes	*E. coli*/other cells	Expressed *Vitreoscilla* hemoglobin gene to improve growth rate and product formation under oxygen-limiting conditions	Khosla and Bailey, 1988 (*120*); Bailey et al., 1990 (*121*); Khosla and Bailey, 1991 (*122*)
Improved growth rate	*Aspergillus nidulans*	Overproduction of glyceraldehyde-3-phosphate dehydrogenase	Hanegraaf et al., 1991 (*123*)
Cell lysis	*E. coli*	ϕX174 lysis gene on temperature-sensitive promotor; lyse cells to simplify purification of intracellular proteins by raising temperature from 30 to 40°C	Dabora et al., 1989 (*124*)
Flocculation	*E. coli*	Used expression of pilin to cause flocculation; in conjunction with recycle reactor can stably mainain plasmids for protein production	Ogden and Davis, 1991 (*125*)
Resistance to methylglyoxal and heavy metals	*Saccharomyces cerevisiae*, *E. coli*	Overexpressed gene from *S. cerevisiae*	Murata et al., 1985 (*126*)

Table 6 (*continued*)

Improvement of *Rhizobium* inoculants	*Rhizobium*	Review article; improved nodulation, nitrogen fixation, carbon transport, substrate utilization, and antibiotic production	Paau et al., 1991 (*127*)
Metal removal	*E. coli*	Cloned metallothionein gene from human	Romeyer et al., 1988 (*128*)
Salt and freezing tolerance	*E. coli*	Overexpressed a proline-containing peptide	Bülow and Mosbach, 1991 (*129*)
Bioluminescence-based detection of organo-mercury, cadmium, lead, and chromate	Various hosts	*lux* operon of *Xenorhabdus luminescens* fused to specific regulatory genes	Rosson, 1992 (*130*)
Sensitivity to (pro)mutagenic chemicals	*Streptomyces griseus*	Added kanamycin-resistance/sensitivity gene for easy detection of point mutations and frameshift mutations	Buchholz et al., 1992 (*131*)
Drought and salt tolerance	Plant cells	Proposed expression of choline mono-oxygenase and betaine dehydrogenase for production of glycine betaine, an osmoprotectant	McCue and Hanson, 1990 (*132*)
Resistance to fungal pathogens	Tobacco plants	Constitutively expressed a bean chitinase gene to enhance resistance to the fungal pathogen *Rhizoctonia solani*	Broglie et al., 1991 (*133*)
Improved secretion of tPA	Chinese hamster ovary (CHO) cells	Used antisense RNA to reduce the level of endogenous GRP78 (a glucose-regulated protein)	Dorner et al., 1988 (*134*)
Alteration of terminal glycosylation sequences; potential alteration of biological recognition	Chinese hamster ovary (CHO) cells	Expression of β-galactoside α 2,6 sialytransferase	Lee et al., 1989 (*135*)

Increased stable accumulation of factor VIII	Chinese hamster ovary (CHO) cells	Co-expression of von Willebrand factor with factor VIII	Kaufman et al., 1989
Production of "essential" amino acids	Mammalian cells	Review and analysis of possibility of expression of fungal and bacterial lysine and threonine biosynthetic pathways in mammalian cells	Ress et al., 1990 (*137*)
Resistance to exogenous methylglyoxal	Chinese hamster ovary (CHO) cells	Expressed *Pseudomonas putida* glyoxalase I in CHO cells	Cameron et al., 1991 (*138*)
Proliferation response to epidermal growth factor (EGF)	B82 mouse fibroblast L cells; NR6 cells	Site-directed mutagenesis of EGR receptor; such modifications may possibly reduce serum growth factor requirements of cells; also design of cell growth control	Lauffenberger et al., 1991 (*139*); Starbuck and Lauffenburger, 1992, (*140*)
Glucose-stimulated insulin secretion and glucose regulation of insulin biosynthesis	Human anterior pituitary cell line AtT-20ins	Expression of GLUT-2, a high-capacity glucose transporter as first step in creating an "artificial β cell"	Hughes et al., 1992 (*141*)

Several examples given in Table 6 are of biomedical significance and fit the notion of cellular engineering as described by Nerem (*8*). One is the modification of the epidermal growth factor receptors on mammalian cells in order to modify the proliferation response. An implication of modifying regulatory receptor/ligand interactions is that it may be possible to reduce the requirement for growth factor by mammalian cells grown in culture (*139*). A second example is the engineering of a human pituitary cell line to have the properties of an insulin-secreting β cell (*141*).

TOOLS FOR CELLULAR AND METABOLIC ENGINEERING

Cellular engineering and metabolic engineering as defined in the Introduction and as illustrated by the examples in Tables 2–6 draw on a wide range of disciplines and technologies. It is probably impossible to summarize all the tools needed to engineer a cell or an organism successfully. However, there are some broad classes of tools that are needed for this work and that will become more important as these fields develop. They include molecular biology tools needed to construct genes and modify organisms, analytical chemistry and measurement tools needed to characterize modified cells, and mathematical and computation tools needed to model, design, and predict the properties of modified organisms. Each of these areas has a vast literature. Highlights and key developments will be presented here.

Molecular Biological Tools

Nearly all of the tools and techniques of genetic engineering are needed for cellular and metabolic engineering. Some tools that have been specifically developed for metabolic engineering are:

1. Transformation systems for industrially important chemical-producing microorganisms, such as *Corynebacterium, C. acetobutylicum* (*59*), and *Micromonospora* (*30*).
2. Special vectors and promoters, such as the excision vector used in the Backman phenylalanine process for the excision of the tyrosine biosynthetic gene to create a tyrosine auxotroph during the final stages of the phenylalanine fermentation (*40,42*) and the regulated promoter used in the 2-KLG process (*81*).
3. Methods for stabilizing cloned genes, such as the integration of the genes into the chromosome of *Pseudomonas aeruginosa* (*99*), cyanobacteria (in a wide-ranging patent on metabolic engineering by Szalay and Williams [*143*]), and *E. coli* (*23*).
4. Markers for the tracing and analysis of metabolic pathways, such as the blue-black markers for *Streptomycetes* (*144*).

Many additional tools will be developed as more of the work in genetic engineering moves from the overproduction of proteins to the engineering of new pathways and new cell properties.

Analytical Chemistry and Measurement Tools

As with the molecular biological tools, any of existing analytical techniques are of critical importance for cellular and metabolic engineering. Metabolic engineering is leading to a resurgence of interest in metabolic pathways; many of the classical techniques for studying pathways, including mass balancing, isotope labeling, and analysis of blocked mutants, are needed. Also, the traditional enzyme assay will continue to be important. However, noninvasive real-time analyses, such as NMR (*54,145*) and flow cytometry, will increase in importance, and new methods will be needed.

Mathematical and Computational Tools

Most of the few existing mathematical and computational tools for metabolic engineering involve information management and systems analysis. Metabolic engineering requires both genetic information, and information on the biochemistry of the pathways and the physiology of the host organism. DNA data bases and computer programs are widely available, but currently do not link DNA data bases with information on metabolism. Recently, Mavrovouniotis (*146*) has developed a group contribution method for estimating standard Gibbs energies of biochemical compounds in aqueous solution, and Karp (*147*) has constructed a computer knowledge base of 981 biochemical compounds, which may help to close this gap.

Cells and organisms are complex integrated systems. Even relatively simple analyses, such as the calculation of the maximum theoretical yield of a metabolite, are difficult. The structure or topology of the pathway greatly influences the yield. Cooney and Acevedo (*148*) have shown how various differences in metabolism (and topological constraints) influence the theoretical yield of penicillin. Papoutsakis (*149*) later outlined the theoretical background of yield analysis and applied it to the acetone-butanol fermentation.

Seressiotis and Bailey (*150,151*) developed an artificial intelligence (AI) program, Metabolic Pathway Synthesis (MPS), for the systematic synthesis of biochemical pathways. MPS generates all the possible pathways from a substrate to a product, but does not balance cofactors. Mavrovouniotis et al. (*152*) developed an AI program for computer-aided synthesis of biochemical pathways that combines elements of pathway synthesis with the calculation of maximum theoretical yields. The program gives all potential routes from the substrate to the product, given a set of appropriate stoichiometric constraints.

The identification of kinetic constraints has been investigated extensively over the last 20 years. Savageau and colleagues (*153–155*) developed Biochemical Systems Theory (BST) to represent the kinetics of biochemical networks in a power law formulation. Kacser and Burns (*156*) and Heinrich et al. (*157*) independently developed Metabolic Control Theory (MCT) to handle similar problems. Although Savageau and his colleagues have shown BST and MCT are mathematically equivalent (*158*), there is still debate about which theory is better for the description of in vivo dynamics (*159*). In both approaches, the kinetic data of a given biochemical network are transformed into a mathematical model, and control coefficients are determined. Control coefficients are an index of the relative importance of a given enzyme to the desired flux around a steady state. The enzyme with the highest control coefficient in a given biochemical network is the rate-limiting step. The concept of control coefficients has been extended to the sensitivity, which is not restricted around a steady state and is a function of time. Malmberg and Hu (*160*) have applied the concept of sensitivity to identify the rate-limiting step in cephalosporin biosynthesis.

Although the above approaches are mathematically sound, the utility of these approaches in metabolic engineering is still limited. This is because the accuracy of the prediction depends heavily on the accuracy of the kinetic rate expressions and the experimental data. Palsson and Lee (*161*) have pointed out that the analysis of an incomplete metabolic model can be misleading and is not indicative of the behavior of the full model. Since most biochemical networks are extremely complicated and most kinetic data are obtained in vitro, the accuracy of sensitivity analysis is doubtful. Liao and Lightfoot (*162*) have proposed the concept of the characteristic reaction path (CPR) to estimate the in vivo kinetics. Recently, Delgado and Liao (*163*) have proposed a method to identify rate-limiting steps without kinetic parameters. Stephanopoulos and Vallino (*164*) have proposed the concept of network rigidity to determine the in vivo metabolic control in a semiempirical way. All these methods await testing in a real metabolic engineering model system. Many of issues relating to metabolic control analysis have recently been reviewed by Liao and Delgado (*165*).

The above theories and analyses are directed toward metabolic engineering. Additional tools and approaches are needed for the analysis of aspects of cellular engineering. An example is the analysis of receptor/ligand interactions developed by Starbuck and Lauffenburger (*140*).

GUIDELINES AND GENERAL CONSIDERATIONS

The development of better analytical techniques and mathematical and computational tools is important for the future of cellular and metabolic engineering. As stated by Bailey (*1*), we need to move "toward a

science of metabolic engineering." The need for more science is supported by the results of some of the best examples of metabolic engineering. For example, in 2-KLG production, the use of a regulatable promoter system gives better results than a constitutive system (*81*). For phenylalanine, the overproduction of some enzymes in the pathway is detrimental to product formation (*42*). However, the successful practitioner also needs to understand the "art" of cellular and metabolic engineering. A number of guidelines for the selection of the host organism and the selection of the pathway are given below. The fact that cellular and metabolic engineering are iterative processes, drawing on many disciplines, must be reemphasized.

One of the first decisions that needs to be made in metabolic engineering is host selection. Many factors must be considered in making this decision. These include the availability of cloning vectors and the ease of cloning (*15*), pathogenicity (*16*), substrate range (*15,16,18*), environmental "hardiness" (*20,22*), the presence of key intermediates (*79,81*), the availability of information on the physiology and genetics of the organism (*42*), the availability of regulatory mutants (*44,68*), the presence of deficiencies in intraplasmid recombination (*97*), and the ability to utilize inexpensive sources of nutritional supplements. For production of food or food additives, the use of generally recognized as safe (GRAS) organisms is desirable. *E. coli* meets many of the above requirements and is the organism of choice for at least the initial stages of many metabolic engineering projects (*42*). One of the most extensive published investigations of host properties is the analysis of lactobacilli as potential hosts for ethanol-producing genes (*166*). In this work, 31 strains were tested for ethanol tolerance and carbohydrate metabolism.

Another key decision is the source of the enzymes for pathway construction. For example, a variety of organisms possess pyruvate decarboxylase, but the *Z. mobilis* enzyme was selected for the ethanol pathway engineering work (*22*). Factors that influence gene selection include the availability of the gene, the kinetic properties of the enzyme (*21*), the temperature stability of the enzyme (*11*), and the need to have unique technology for patent purposes (e.g., compare [*81*] with [*82*]). Other factors of potential importance, but that have only been addressed peripherally in the examples cited in this article, are the complexity of the enzyme, specific cofactor requirements, the regulatory properties of the enzymes (*42*), and enzyme-enzyme interactions.

Many authors have pointed out the iterative and multidisciplinary nature of cellular and metabolic engineering. Bailey describes some of the "potential and perils of rational design" in completing the metabolic engineering cycle (*1*). The perils include use of industrial strains rather than laboratory strains, the lack of good analytical methods, the lack of design principles, and the high probability of unanticipated cell responses to modifications. McCue and Hanson (*132*) summarize the iterative approach

in a flow chart that is specifically aimed at modifying plant stress resistance, but that is generally applicable to cellular and metabolic engineering. Mathematical models and computer simulations are also important steps in the iterative process (*87*). The importance of combining the knowledge of areas such as genetic engineering, microbiology, chemical engineering, fermentation, and processing, is emphasized by Murata and Kimura (*48*) and Backman et al. (*42*).

FUTURE DIRECTIONS AND CONCLUSIONS

The fields of cellular and metabolic engineering are less than 20 years old (dating from the 1974 patent of Chakrabarty et al. [*109*]). Although much has been accomplished during this time, the technology is far from mature. One area that is likely to have a great impact on metabolic engineering is the use of catalytic antibodies to create novel metabolic activities. A catalytic antibody was recently used to complement an insertion mutation in the structural gene for chorismate mutase in yeast (*167*). Another area is the use of fusion proteins for the catalysis of sequential or coupled metabolic reactions. *E. coli*-containing β-galactosidase-galactokinase fusion proteins with short linkers grew faster on lactose in the presence of galactose dehydrogenase than cells containing the same fusion protein, but with longer linkers (*168*). This indicates that the proximity of enzymes to one another has an effect on metabolism. Fused enzymes have also been used in *S. cerevisiae* for the whole-cell hydroxylation of progesterone to 17 α-hydroxyprogesterone (*169*). The "programming" of polyketide synthases to make hybrid complexes capable of catalyzing the synthesis of "hybrid" polyketide-derived carbon chains (*170*) is a further extension of metabolic engineering. Some novel aspects of cellular engineering have been suggested for the improvement of cells for the degradation of toxic wastes. They include the design of transport systems for toxic compounds and the construction of cells with the ability to move toward poorly diffusible compounds (*4*).

An understanding of cells as integrated systems will become increasingly important. Mittenthal et al. (*171*) have recently described a theory of organization for the bacterium *E. coli* in a paper entitled "Designing bacteria." Savageau (*172*) has called for the development of integrative biology. The needs of cellular and metabolic engineering may well be the driving force for such a development.

The purpose of this article has been to provide a broad overview of cellular end metabolic engineering. These fields will continue to expand and to be important in their own right. They will also provide an important foundation for the development of even more complex technologies, such as tissue engineering, gene therapy, and whole-organism engineering.

ACKNOWLEDGMENTS

The authors thank Sarah Castello of the University of Wisconsin-Madison Biotechnology Center for assistance with bibliographic data base searching and Winnie Teng for general assistance. We also thank the UW Bioprocess and Metabolic Engineering Consortium/BioTAP for financial support.

REFERENCES

1. Bailey, J. E. (1991), *Science* **252,** 1668–1675.
2. MacQuitty, J. J. (1988), *ACS Symposium Series* **362,** 11–29.
3. Tong, I.-T., Liao, H. H., and Cameron, D. C. (1991), *Appl. Environ. Microbiol.* **57,** 3541–3546.
4. Timmis, K. N., Rojo, F., and Ramos, J. L. (1988), in *Environmental Biotechnology*, Omenn, G. S., ed., Plenum Press, New York, pp. 61–79.
5. Chakrabarty, A. M. (1992), University of Illinois-Chicago, personal communication.
6. Kellogg, S. T., Chatterjee, D. K., and Chakrarty, A. M. (1981), *Science* **214,** 1133–1135.
7. Kanayama, H., Sode, K., Yamamoto, T., and Karube, I. (1988), *Biotechnol. Genet. Eng. Rev.* **6,** 379–401.
8. Nerem, R. M. (1991), *Ann. Biomed. Eng.* **19,** 529–545.
9. Draths, K. M. and Frost, J. W. (1990), *J. Am. Chem. Soc.* **112,** 9630–9632.
10. Draths, K. M. and Frost, J. W. (1990), *J. Am. Chem. Soc.* **112,** 1657–1659.
11. Wood, B. E. and Ingram, L. O. (1992), *Appl. Environ. Microbiol.* **58,** 2103–2110.
12. Rogers D. T. and Szostak, J. W. (1987), Yeast strains. PCT Int. Appl. WO 87/3006 A1; *Chem. Abstr.* **107,** 234858r.
13. Fukaya, M., Tayama, K., Tamaki, T., Tagami, H., Okumura, H., Kawamura, Y., and Beppu, T. (1989), *Appl. Environ. Microbiol.* **55,** 171–176.
14. Brau, B. and Sahm, H. (1986), *Arch. Microbiol.* **144,** 296–301.
15. Tolan, J. S. and Finn, R. K. (1987), *Appl. Environ. Microbiol.* **53,** 2033–2038.
16. Tolan, J. S. and Finn, R. K. (1987), *Appl. Environ. Microbiol.* **53,** 2039–2044.
17. Ingram, L. O. and Clark, D. P. (1991), Ethanol production using engineered *E. coli*. US Patent 5,028,539.
18. Ingram, L. O., Conway, T., Clark, D. P., Sewell, G. W., and Preston, J. F. (1987), *Appl. Environ. Microbiol.* **53,** 2420–2425.
19. Ingram, L. O. and Conway, T. (1988), *Appl. Environ. Microbiol.* **54,** 397–404.
20. Alterthum, F. and Ingram, L. O. (1989), *Appl. Environ. Microbiol.* **55,** 1943–1948.
21. Ingram, L. O., Alterthum, F., Ohta, K., and Beall, D. S. (1990), *Dev. Ind. Microbiol.* **31,** 21–30.
22. Ingram, L. O., Conway, T., and Alterthum, F. (1991), Ethanol production by *Escherichia* strains co-expressing *Zymomonas* PDC and ADH genes. US Patent 5,000,000.

23. Ohta, K., Beall, D. S., Mejia, J. P., Shanmugam, K. T., and Ingram, L. O. (1991), *Appl. Environ. Microbiol.* **57,** 893–900.
24. Ohta, K., Beall, D. S., Mejia, P. J., Shanmugam, K. T., and Ingram, L. O. (1991), *Appl. Environ. Microbiol.* **57,** 2810–2815.
25. Feldmann, S., Sprenger, G. A., and Sahm, H. (1989), *Appl. Microbiol. Biotechnol.* **31,** 152–157.
26. Mermelstein, L. D., Welker, N. E., Bennett, G. N., and Papoutsakis, E. T. (1992), *Biotechnology* **10,** 190–195.
27. Cox, K. L., Fishman, S. E., Hershberger, C. L., and Seno, E. T. (1987), Construction of genetically engineered microorganism having improved antibiotic-producing ability. Eur. Pat. Appl. EP 238323 A2; *Chem. Abstr.* **108,** 54455u.
28. Chen, C. W., Lin, H.-F, Kuo, C. L., Tsai, H.-L., and Tsai, J. F.-Y. (1988), *Biotechnology* **6,** 1222–1224.
29. Skatrud, P. L., Tietz, A. J., Ingolia, T. D., Cantwell, C. A., Fisher, D. L., Chapman, J. L., and Queener, S. W. (1989), *Biotechnology* **7,** 477–485.
30. Rothstein, D. M., Love, S.F., and Baum, E. Z. (1991), Genetic system for micromonospora. US Patent 5,024,948.
31. Hutchinson, C. R. (1992), *Pharm. Technol.* **16,** 22.
32. Beckmann, R. J., Cox, K. L., Rao, R. N., Richardson, M. A., and Seno, E.T. (1992), Macrolide biosynthetic genes for use in streptomyces and other organisms. US Patent 5,098,837.
33. Ifuku, O., Haji, S., Kishimoto, J., Sakamoto, T., and Yenagi, M. (1986), High-yield biotin expression vector for *Escherichia coli.* Jpn. Kokai Tokkyo Koho JP 61/202686 A2; *Chem. Abstr.* 1987, **106,** 170235e.
34. Katsumata, R., Mizukami, T., and Oka, T. (1986), L-Lysine manufacture by recombinant DNA technology. Jpn. Kokai Tokkyo Koho JP 61/209597 A2: *Chem. Abstr.* 1987, **106,** 174689d.
35. Kisumi, M. and Takagi, T. (1987), Method for producing L-aspartic acid. US Patent 4,656,136.
36. Sugita, T. and Komatsubara, S. (1989), *Appl. Microbiol. Biotechnol.* **30,** 290–293.
37. Katsumata, R. and Yokoi, H. (1988), Transformation of *Corynebacterium* and *Brevibacterium* with plasmids encoding arginine biosynthesis enzymes and arginine manufacture with the transformants. Eur. Pat. Appl. EP 261627 A2; *Chem. Abstr.* 1989, **110,** 129896r.
38. Katsumata, R., Yokoi, H., Kino, K. (1988), L-Glutamic acid and L-proline, their recombinant manufacture with *Corynebacterium* and *Brevibacterium.* Jpn. Kokai Tokkyo Koho JP 63/119688, A2; *Chem. Abstr.* 1989, **110,** 113201z.
39. Katsumata, R., Yokoi, H., Kino, K. (1988), L-Glutamic acid and its manufacture with recombinant *Corynebacterium* and *Brevibacterium.* Jpn. Kokai Tokkyo Koho JP 63/102692, A2; *Chem. Abstr.* 1988, **109,** 188807q.
40. Backman, K. C. and Balakrishnan, R. (1988), Controlled gene excision. US Patent 4,743,546.
41. Backman, K. C. (1989), Method of biosynthesis and cells therefor. US Patent 4,839,286.
42. Backman, K., O'Connor, M. J., Maruya, A., Rudd, E., McKay, D., Balakrishnan, R., Radjai, M., DiPasquantonio, V., Shoda, D., Hatch, R., and Venkatasubrubramanian, K. (1990), *Ann. NY Acad. of Sci.* **589,** 16–24.

43. Lee, S. B., Won, C. H., Park, C., and Lim, B. S. (1991), Method for production of L-phenylalanine by recombinant *E. coli.* US Patent 5,008,190.
44. Ikeda, M. and Katsumata, R. (1992), *Appl. Environ. Microbiol.* **58,** 781–785.
45. Pollock, T. J. and Thorne, L. (1988), Enhanced manufacturing of xanthan gum with genetically modified *Xanthomonas.* Eur. Pat. Appl. EP 287363; *Chem. Abstr.* 1989, **105,** 93587z.
46. Ben-Bassat, A., Calhoon, R. D., Fear, A. L., Gelfand, D. H., Meade, J. H., Tal, R., Wong, H., and Bensiman, M. (1990), The cellulose synthase operon of *Acetobacter:* cloning and expression. PCT Int. Appl. WO 9012098 A2 18 Oct 1990; *Chem. Abstr.* 1991, **115,** 200336u.
47. Wong, H. C., Fear, A. L., Calhoon, R. D., Eichinger, G. H., Mayer, R., Amikam, D., Benziman, M., Gelfand, D. H., Meade, J. H., Emerick, A. W., Bruner, R., Ben-Bassat, A., and Tal, R. (1990), *Proc. Natl. Acad. Sci. USA* **87,** 8130–8134.
48. Murata, K. and Kimura, A. (1990), *Biotech. Adv.* **8,** 59–96.
49. Shiomi, N. and Fukuda, H. (1992), Gene capable of enhancing *S*-adenosyl-L-methionine accumulation and process for producing *S*-adenosyl-L-methionine using the same. US Patent 5,100,786.
50. Frost, J. W., Reimer, L. M., Draths, K. M., Pompliano, D. L., and Conely, D. L. (1990), in *Biocatalysis,* Abramowicz, D. A., ed., Van Nostrand, New York, pp. 93–113.
51. Draths, K. M., Pompliano, D. L., Conley, D. L., Frost, J. W., Berry, A., Disbrow, G. L., Staversky, R. J., and Lievense, J. C. (1992), *J. Am. Chem. Soc.* **114,** 3956–3962.
52. Dunahay, T. G., Jarvis, E. E., Zeiler, K. G., Roessler, P. G., and Brown, L. M. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 331–339.
53. Beall, D. S., Ohta, K., and Ingram, L. O. (1991), *Biotechnol. Bioeng.* **38,** 296–303.
54. Diaz-Ricci, J. C., Hitzmann, B., and Bailey, J. E. (1991), *Biotechnol. Prog.* **7,** 305–310.
55. Diaz-Ricci, J. C., Tsu, M., and Bailey, J. E. (1991), *Biotechnol. Bioeng.* **39,** 59–65.
56. Lawford, H. G., and Rousseau, J. D. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 185–204.
57. Lawford, H. G. and Rousseau, J. D. (1991), *Appl. Biochem. Biotechnol.* **28/29,** 221–236.
58. Ohta, K., Alterhum, F., and Ingram, L. O. (1990), *Appl. Environ. Microbiol.* **56,** 463–465.
59. Papoutsakis, E. T. and Bennet, G. N. (1991), in *Clostridia and Biotechnology,* Wood, D. R., ed., Butterworth, Oxford, UK.
60. Hopwood, D. A., Malpartida, F., Kieser, H. M., Ikeda, H., Duncan, J., Fujii, I., Rudd, B. A. M., Floss, H. G., and Omura, S. (1985), *Nature (Lond.),* **314,** 642–644.
61. Stanzak, R., Matsushima, P., Baltz, R. H., and Rao, R. N. (1986), *Biotechnology* **4,** 229–232.
62. Weber, J. M., Leung, J. O., Swanson, S. J., Idler, K. B., and McAlpine, J. B. (1991), *Science* **252,** 114–117.
63. Epp, J. K., Huber, L. B., Turner, J. R., Goodson, T., and Schoner, B. E. (1989), *Gene* **85,** 293–301.

64. Smith, D. J., Burnham, M. K. R., Edwards, J., Earl, A. J., and Turner, G. (1991), *Biotechnology* **8,** 39–41.
65. Slater, S. C., Voige, W. H., and Dennis, D. E. (1988), *J. Bacteriol.* **170,** 4431–4436.
66. Peoples, O. P. and Sinskey, A. J. (1990), in *Novel Biodegradable Microbial Polymers,* Dawes, E. A., ed., Kluwer Academic Publishers, Netherlands, pp. 191–202.
67. Timm, A., Byrom, D., and Steinbuchel, A. (1990), *Appl. Microbiol. Biotechnol.* **33,** 296–301.
68. Slater, S., Gallaher, T., and Dennis, D. (1992), *Appl. Environ. Microbiol.* **58,** 1089–1094.
69. Poirier, Y., Dennis, D. E., Klomparens, and Somerville, C. (1992), *Science* **256,** 520–523.
70. Hassler, R. A. and Doherty, D. H. (1990), *Biotechnol. Prog.* **6,** 182–187.
71. della-Cioppa, G., Garger, S. J., Sverlow, G. G., Turpen, T. H., and Grill, L. K. (1990), *Biotechnology* **8,** 634–638.
72. Ensley, B. D., Ratzkin, B. J., Osslund, T. D., Simon, M. J., Wackett, L. P., and Gibson, D. T. (1983), *Science* **222,** 167–169.
73. Ensley, B. D. (1985), Microbial production of indigo. US Patent 4,520,103.
74. Mermod, N., Harayama, S., and Timmis, K. N. (1986), *Biotechnology* **4,** 321–324.
75. Keil, H., Saint, C. M., and Williams, P. A. (1987), *J. Bacteriol.* **169,** 764–770.
76. Stephens, G. M., Sidebotham, J. M., Mann, N. H., and Dalton, H. (1989), *FEMS Microbiol. Lett.* **57,** 295–300.
77. Hart, S. and Woods, D. R. (1992), *J. Gen. Microbiol.* **138,** 205–209.
78. Hart, S., Koch, K. R., and Woods, D. R. (1992), *J. Gen. Microbiol.* **138,** 211–216.
79. Anderson, S., Marks, C. B., Lazarus, R., Miller, J., Stafford, K., Seymour, J., Light, D., Rastetter, W., and Estell, D. (1985), *Science* **230,** 144–149.
80. Lazarus, R. A., Seymour, J. L., Stafford, R. K., Dennis, M. S., Lazarus, M. G., Marks, C. B., and Anderson, S. (1990), in *Biocatalysis,* Abramowicz, D. A., ed., Van Nostrand Reinhold, New York, pp. 135–155.
81. Anderson, S., Lazarus, R. A., Miller, H. I., and Stafford, R. K., (1991), Metabolic pathway engineering to increase production of ascorbic acid intermediates. US Patent 5,032,514.
82. Hardy, K., van de Pol, H., Grindley, J., and Payton, M. A. (1990), Production of a vitamin C precursor using genetically modified organisms. US Patent 4,945,052.
83. Goelling, D. and Stahl, U. (1988), *Appl. Environ. Microbiol.* **54,** 1889–1891.
84. Katsumata, R. and Ikeda, M. (1988), Manufacture of tyrosine with recombinant tryptophan-producing *Corynebacterium* or *Brevibacterium.* Eur. Pat. Appl. EP 263515 A2, *Chem. Abstr.* 1988, **109,** 228741x.
85. Laeufer, A., Gassen, H. G., Flachmann, R., Hoeke, H., Hoeltman, W., Kunz, N., Stadelhofer, J., and Seifert, J. (1988), Cloning of microbial *nadA* and *nadB* genes and quinolinic acid manufacture with microorganisms transformed with plasmids containing these genes. Ger. Ofen. DE 3703255 A1; *Chem. Abstr.* 1989, **110,** 52336y.
86. Tong, I.-T., and Cameron, D. C. (1992), *Appl. Biochem. Biotech.* **34/35,** 149–159.

87. Tong, I.-T. (1992), Microbial production of 1,3-propanediol in *Escherichia coli:* a model system for metabolic engineering. Ph.D. Thesis, University of Wisconsin-Madison.
88. Draths, K. M. and Frost, J. W. (1991), *J. Am. Chem. Soc.* **113,** 9361–9363.
89. Hallborn, J., Walfridsson, M., Airaksinen, U., Ojamo, H., Hanhn-Hagerdal, B., Penttila, M., and Keranen, S. (1991), *Biotechnology* **9,** 1090–1095.
90. Favre-Bulle, O., Schouten, T., Kingma, J., and Withholt, B. (1991), *Biotechnology* **9,** 367–371.
91. Oakes, J. V., Skewmaker, C. K., and Stalker, D. M. (1991), *Biotechnology* **9,** 982–986.
92. Saito, K., Yamazaki, M., Kawaguchi, A., and Murakoshi, I. (1991), *Tetrahedron.* **47,** 5955–5968.
93. Misawa, N., Yamano, S., and Ikenaga, H. (1991), *Appl. Environ. Microbiol.* **57,** 1847–1849.
94. Ausich, R. L., Proffitt, J. H., and Yarger, J. G. (1991), Biosynthesis of carotenoids in genetically engineered hosts. International Patent Application International Application Number: PCT/US91/01458; *Chem. Abstr.* 1992, **116,** 35644d.
95. Voelker, T. A., Worrell, A. C., Anderson, L., Bleibaum, J., Fan, C., Hawkins, D. J., Radke, S. E., and Davies, H. M. (1992), *Science* **257,** 72–74.
96. Picataggio, S., Rohrer, T., Deanda, K., Lanning, D., Reynolds, R., Mielenz, J., and Eirich, L. D. (1992), *Biotechnology* **10,** 894–898.
97. Chater, K. F. (1990), *Biotechnology* **8,** 115–121.
98. Tsai, H.-J., and Sandine, W. E. (1987), *J. Indust. Microbiol.* **2,** 25–33.
99. Koch, A. K., Reiser, J., Kappeli, O., and Fiechter, A. (1988), *Biotechnology* **6,** 1335–1339.
100. Fu, J.-F. and Tseng, Y.-H. (1990), *Appl. Environ. Microbiol.* **56,** 919–923.
101. Pries, A., Steinbuchel, A., and Schegel, H. G. (1990), *Appl. Microbiol Biotechnol.* **33,** 410–417.
102. Buchholz, S. E. and Eveleigh, D. E. (1990), *Biotech. Adv.* **8,** 547–581.
103. Kumar, V., Ramakrishnan, S., Teeri, T. T., Knowles, J. K., C., and Hartley, B. S. (1992), *Biotechnology* **10,** 82–85.
104. Burchhardt, G. and Ingram, L. O. (1992), *Appl. Environ. Microbiol.* **58,** 1128–1133.
105. Tsunekawa, H., Azuma, S., Okabe, M., Okamoto, R., and Aiba, S. (1992), *Appl. Environ. Microbiol.* **58,** 2081–2088.
106. Romano, A. H. (1986), *Trends in Biotechnology* **4,** 207–213.
107. Sarthy, A. V., McConaughy, B. L., Lobo, Z., Sundstrom, J. A., Furlong, C. E., and Hall, B. D. (1987), *Appl. Environ. Microbiol.* **53,** 1996–2000.
108. Ho, N. W. Y. (1992), Genetic engineering of yeast for the conversion of xylose to ethanol fuel and chemicals. Abstracts of Papers, 1992 Annual Meeting, American Institute of Chemical Engineers, Miami Beach, Florida, AIChE, New York, 154d.
109. Chakrabarty, A. M. (1974), Microorganisms having multiple compatible degradative energy-generating plasmids and preparation thereof. US Patent 3,813,316.
110. Rojo, F., Pieper, D. H., Engesser, K.-H., Knackmuss, H.-J., and Timmis, K. N. (1987), *Science* **238,** 1395–1398.

111. Ramos, J. L., Wasserfallen, A., Rose, K., and Timmis, K. N. (1987), *Science* **235,** 593–596.
112. Winter, R. B., Yen, K.-M., and Ensley, B. E. (1989), *Biotechnology* **7,** 282–285.
113. Mondello, F. J. (1989), *J. Bacteriol.* **171,** 1725–1732.
114. Adams, R. H., Huang, C.-M., Higson, F. K., Brenner, V., and Focht, D. D. (1992), *Appl. Environ. Microbiol.* **58,** 647–654.
115. Hutchinson, C. R. (1987), *Appl. Biochem. Biotechnol.* **16,** 169–189.
116. Hutchinson, C. R. (1988), *Med. Res. Rev.* **8,** 557–567.
117. Amato, I. (1991), *Science* **253,** 1214.
118. Windass, J. D., Worsey, M. J., Pioli, E. M., Barth, P. T., Atherton, K. T., Dart, E. C., Byrom, D., and Senior, P. J. (1980), *Nature (Lond.)* 287: 396–401.
119. Senior, P. J. and Windass, J. (1980), *Biotechnol. Lett.* **2,** 205–210.
120. Khosla, C. and Bailey, J. E. (1988), *Nature (Lond.)* **331,** 633–635.
121. Bailey, J. E., Birnbaum, S., Galazzo, J. L., Khosla, C., and Shanks, J. V. (1990), *Ann. NY Acad. of Sci.* **589,** 1–15.
122. Khosla, C. S. and Bailey, J. E. (1991), Enhancement of cell growth by expression of cloned hemoglobin gene. US Patent 5,049,493.
123. Hanegraaf, P. P. F., Punt, P. J., van den Hondel, C. A. M., Dekker, J., van Verseveld, H. W., and Stouthamer, A. H. (1991), *Appl. Microbiol. Biotechnol.* **34,** 765–771.
124. Dabora, R. L., Eberiel, D. T., and Cooney, C. L. (1989), *Biotechnol. Lett.* **11,** 845–850.
125. Ogden, K. L. and Davis, R. H. (1991), *Biotechnol. Bioeng.* **37,** 325–333.
126. Murata, K., Fukudu, Y., Shimosaka, M., Watanabe, K., Saikusa, T., and Kimura, A. (1985), *Appl. Environ. Microbiol.* **50,** 1200–1207.
127. Paau, A. S. (1991), *Biotech. Adv.* **8,** 173–184.
128. Romeyer, F. M., Jacobs, F. A., Masson, L., Hanna, Z., and Brousseau, R. (1988), *J. Biotechnol.* **8,** 207–220.
129. Bülow, L. and Mosbach, K. (1991), *Gene* **109,** 125–129.
130. Rosson, R. (1992), Biotechnical Resources, Inc., Manitowoc, WI, personal communication.
131. Buchholz, S. E., Omer, C. A., Viitanen, P. V., Sariaslani, F. S., and Stahl, R. G., Jr. (1992), *Appl. Biochem. Biotechnol.* **32,** 149–158.
132. McCue, K. F. and Hanson, A. D. (1990), *Trends in Biotechnol.* **8,** 358–362.
133. Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C. J., and Broglie, R. (1991), *Science* **254,** 1194–1199.
134. Dorner, A. J., Krane, M. G., and Kaufman, R. J. (1988), *Mol. Cell. Biol.* **8,** 4063–4070.
135. Lee, E. U., Roth, J., and Paulson, J. C. (1989), *J. Biol. Chem.* **264,** 13,848–13,855.
136. Kaufman, R. J., Wasley, L. C., Davies, M. V., Wise, R. J., Israel, D. I., and Dorner, A. J. (1989), *Mol. Cell. Biol.* **9,** 1233–1242.
137. Rees, W. D., Flint, H. J., and Fuller, M. F. (1990), *Biotechnology* **8,** 629–633.
138. Cameron, D. C., Chaplen, F., and Fahl, W. (1991), Influence of glyoxalase I activity on mammalian cells grown in culture. Abstracts of Papers, 1991 Annual Meeting, American Institute of Chemical Engineers, Los Angeles, CA: AIChE, NY; 267d.

139. Lauffenburger, D. A., Starbuck, C., and Wiley, H. S. (1991), Engineering growth factor/receptor processes: effects of EGF receptor trafficking dynamics on cell proliferation responses to EGF. *Proceedings of 1991 US/Japan Conference on Biotechnology*, Tanner, R. D., ed., Springer-Verlag, New York.
140. Starbuck, C. and Lauffenburger, D. A. (1992), *Biotechnol. Prog.* **8,** 132–143.
141. Hughes, S. D., Johnson, J. H., Quaade, C., and Newgard, C. B. (1992), *Proc. Natl. Acad. Sci. USA* **89,** 688–692.
142. Danheiser, S. L. (1992), *Genetic Engineering News* **12,** 1,19,22.
143. Szalay, A. A. and Williams, J. G. K. (1988), Genetic engineering in cyanobacteria. US Patent 4,778,759.
144. Muth, G., Wohlleben, W., Pühler, A., Wöhner, G., and Marquardt, R. (1990), Color marker in streptomycetes plasmids. US Patent 4,963,496.
145. Shanks, J. V., Fernet, M.-D., and Ho, C.-H. (1992), in *Advances in Bioreactor Monitoring*, Wang, N. S., ed., Academic Press, New York, NY.
146. Mavrovouniotis, M. L. (1990), *Biotechnol. Bioeng.* **36,** 1070–1082.
147. Karp, P. D. (1992), *CABIOS* **8,** 347–357.
148. Cooney, C. L. and Acevedo, F. (1977), *Biotechnol. Bioeng.* **19,** 1449–1462.
149. Papoutsakis, E. T. (1984), *Biotechnol. Bioeng.* **26,** 174–187.
150. Seressiotis, A. and Bailey, J. E. (1986), *Biotechnol. Lett.* **8,** 837–842.
151. Seressiotis, A. and Bailey, J. E. (1988), *Biotechnol. Bioeng.* **31,** 587–602.
152. Mavrovouniotis, M. L., Stephanopoulos, G. and Stephanopoulos, G. (1990), *Biotechnol. Bioeng.* **36,** 1119–1132.
153. Savageau, M. A. (1969), *J. Theor. Biol.* **25,** 365–369.
154. Savageau, M. A. (1969), *J. Theor. Biol.* **25,** 370–379.
155. Savageau, M. A. (1970), *J. Theor. Biol.* **26,** 215–226.
156. Kacser, H. and Burns, J. A. (1973), *Symp. Soc. Exp. Biol.* **27,** 65–104.
157. Heinrich, R., Rapoport, S. M., and Rapoport, T. A. (1977), *Prog. Biophys. Molec. Biol.* **32,** 1–82.
158. Savageau, M. A., Voit, E. O., and Irvine, D. H. (1987), *Math. Biosci.* **86,** 127–145.
159. Savageau, M. A. (1992), *J. Theor. Biol.* **154,** 131–136.
160. Malmberg, L. and Hu, W. (1991), *Biotechnol. Bioeng.* **38,** 941–947.
161. Palsson, B. O. and Lee, I.-D. (1992), University of Michigan, personal communication.
162. Liao, J. C. and Lightfoot, E. N. (1988), *Biotechnol. Bioeng.* **31,** 847–854.
163. Delgado, J. P. and Liao, J. C. (1991), *Biotechnol. Prog.* **7,** 17–20.
164. Stephanopoulos, G. and Vallino, J. J. (1991), *Science* **252,** 1675–1681.
165. Liao, J. C. and Delgado, J. (in press), *Biotechnol. Prog.*
166. Gold, R. S., Meagher, M. M. Hutkins, R., and Conway, T. (1992), *J. Indust. Microbiol.* **10,** 45–54.
167. Tang, Y., Hicks, J. B., and Hilvert, D. (1991), *Proc. Natl. Acad. Sci. USA* **88,** 8784–8786.
168. Bülow, L. and Mosbach, K. (1991), *Trends in Biotechnol.* **9,** 226–231.
169. Shibata, M., Sakaki, T., Yabusaki, Y., Murakami, H., and Ohkawa, H. (1990), *DNA and Cell Biology* **9,** 27–36.
170. Hopwood, D. A. and Khosla, C. (1992), in *Secondary Metabolites: Their Function and Evolution*, Chadick, D. J. and Whelan, J., eds., Wiley Interscience, UK.

171. Mittenthal, J. E., Clarke, B., and Levinthal, M. (1992), in *Thinking About Biology,* Varela, F. and Stein, W., eds., Addison-Wesley, Reading, MA.
172. Savageau, M. A. (1992), in *Fundamentals of Medical Cell Biology,* Bittar, E. E., ed., JAI Press, pp. 45–108.