Developments in metabolic engineering

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The complete sequencing of several microbial genomes has resulted in the increased availability of genes for metabolic engineering. The number of databases and computational tools to deal with this information has also increased. This development has stimulated, and will continue to stimulate, advances in metabolic engineering. Specific recent advances include improvement of pathways for aromatic metabolites, the development of a more complete understanding of the effect of bacterial hemoglobin on cell performance, the development of NMR-based methods for the monitoring of intracellular metabolites and metabolic flux, and the application of metabolic control analysis and metabolic flux analysis to a variety of systems.

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Abbreviations

BST	biochemical systems theory
CHO	Chinese hamster ovary
DAHP	3-deoxy-D-arabino-heptulosonate 7-phosphate
MCA	metabolic control analysis
MFA	metabolic flux analysis
PEP	phosphoenolpyruvate
PHB	polyhydroxybutyrate
PTS	phosphotransferase system
VHb	*Vitreoscilla* hemoglobin

Introduction

The development of recombinant DNA technology has led to the emergence of the field of metabolic engineering, the purposeful and directed modification of intracellular metabolism and cellular properties. Previous work in this field has been the subject of several specific reviews [1–3,4•,5•] and several general reviews [6–8]. The scope of this review is work published in 1995 or later that was not covered by Farmer and Liao [8]. This review is not comprehensive in depth or breadth; rather, it highlights selected examples and emerging trends.

During the past two years, the most significant event for the field of metabolic engineering was the sequencing of several entire microbial genomes. The first completely sequenced genome, for the bacterium *Haemophilus influenzae*, was reported in July 1995 [9••]. Since then, complete genome sequences have been reported for *Mycoplasma genitalium* [10•], *Methanococcus jannaschii* [11•] and *Saccharomyces cerevisiae* [12•]. The publication of the complete genome sequence of *Escherichia coli* is imminent (F Blattner, personal communication). In the past, the field of metabolic engineering has been 'information-limited', that is, progress has been slowed by the lack of gene sequence information. With the availability of several completely sequenced genomes, and many sequenced genes from other organisms, the field is now, to quote Bernhard Palsson of the University of California at San Diego, 'imagination-limited'. The impact of this large and rapidly growing body of DNA sequence information on metabolic engineering has yet to show up in the published literature. However, the availability of such information is a driving force for the development of this field.

This review is organized in a manner similar to that of Cameron and Tong [7]. First, some recent examples of metabolic engineering are described. Then, molecular biological, analytical, and mathematical and computational tools for metabolic engineering are discussed. Finally, some future directions and considerations are presented.

Recent examples of metabolic engineering

Metabolic engineering has been used to improve the production of existing metabolites, enable the production of new metabolites, impart new catabolic activities, and improve cell properties such as 'performance' under suboptimal conditions.

Succinic acid is a metabolite of much interest as a polymer intermediate and as a precursor for chemicals such as 1,4-butanediol and tetrahydrofuran. Millard *et al.* [13•] have demonstrated enhanced succinate production in *E. coli* by the overexpression of phosphoenolpyruvate (PEP) carboxylase. Another metabolite of much commercial interest is lactic acid. Porro *et al.* [14•] expressed a mammalian lactate dehydrogenase in *S. cerevisiae* and, after some fermentation process development, demonstrated significant lactic acid production by the modified organism. Several groups have investigated the metabolic engineering of *Lactococcus lactis* to modify the production of diacetyl, acetoin, and 2,3-butanediol in this organism [15•,16,17]. This system is especially interesting and provides a challenge for metabolic engineering because the product mix is determined by the interplay between anaerobic and aerobic metabolism and between enzymatic and nonenzymatic reactions.

The aromatic amino acids and related compounds are a class of products that have been the subject of much metabolic engineering effort [2]. PEP is an important in-

termediate in these pathways. The common route for glucose transport in *E. coli* is the glucose phosphotransferase system (PTS), in which PEP is consumed during glucose transport. Flores *et al.* [18••] have isolated *E. coli* strains that are able to transport glucose by a system that does not require PEP. These strains, which presumably have more PEP available, were shown to have improved production of 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP), the first intermediate in the common aromatic pathway. In a further study, Gosset *et al.* [19•] combined several factors, including a modified uptake system, and demonstrated a 20-fold increase in carbon commitment to the common aromatic pathway in *E. coli*.

Another area of much metabolic engineering activity is the production of polyhydroxybutyrate (PHB) and other polyhydroxyalkanoates in bacteria, yeast and plants. An novel application of this technology is the metabolic engineering of PHB in cotton. John and Keller [20••] expressed two PHB biosynthetic genes in cotton and demonstrated the production of transgenic cotton fibers containing PHB in the lumen of the fibers. Such fibers exhibited improved insulating characteristics over normal fibers.

An important application of metabolic engineering is the construction of improved organisms for use in the bioremediation of toxic waste [1]. Two recent examples focus on extending the range of substrates that can be catabolized by a single organism. Lee *et al.* [21] report the construction of a hybrid strain of *Pseudomonas putida* that is able to simultaneously mineralize benzene, toluene and p-xylene without the accumulation of metabolic intermediates. Suyama *et al.* [22] constructed a hybrid *Pseudomonas* that is able to grow on a wide range of hydrocarbons and can efficiently degrade trichloroethylene.

A variety of modifications have been investigated to improve cell properties. The most widely researched modification is the overexpression of *Vitreoscilla* hemoglobin (VHb) to improve growth and product formation under suboptimal oxygen levels [3,4•]. Tsai *et al.* [23•] report on the influence of VHb on carbon and energy metabolism in *E. coli*. In a related paper, Tsai *et al.* [24•] also explore the mechanism by which this protein enhances growth and protein production in *E. coli*. Kallio and Bailey [25] report that VHb leads to enhanced growth and secreted protein activity when expressed in *Bacillus subtilis*. In a further study, VHb was overexpressed in *Xanthomonas maltophilia*, an organism of interest for use in bioremediation, and was found to enhance the ability of this organism to degrade benzoic acid [26]. Another protein that has been overexpressed to improve a bioremediation organism is mouse metallothionein. The expression of this protein confers cadmium tolerance and accumulation ability to the cyanobacterium *Synechoccocus* [27]. Finally, Chaplen *et al.* [28•] have overexpressed a bacterial glyoxalase I activity in Chinese hamster ovary (CHO) cells and have shown that these cells have both a lower level of intracellular methylglyoxal, a toxic intermediate, and greater survival ability in colony-forming assays than control cells without enhanced glyoxalase I activity.

Molecular biological tools

Wang and Da Silva [29••] have developed a new approach for the stable expression of multiple genes in yeast. These genes were stable through 100 generations of growth under nonselective conditions. The approach is based on the Ty3 retrotransposon, and allows multiple rounds of gene insertion at nonessential sites in yeast chromosomes. Depending on the integration protocol utilized, it is possible to achieve a specific number of gene insertions with each insertion cycle. Subsequent cycles can be used to insert further copies of the same gene, or copies of different genes, resulting in considerable flexibility for engineering new pathways and cell properties in yeast.

Work has progressed on the development of a transformation system for *Thermoanaerobacterium thermosaccharolyticum* [30•]. This is a potentially important organism for the production of ethanol, 1,2-propanediol and other chemicals; however, because this organism has a branched fermentation pathway, metabolic engineering may be needed to improve the selectivity of the desired product.

Another useful development involves a baculovirus expression vector based on the viral ie1 promoter for protein production during the early stages of insect cell infection [31•]. The utility of this vector lies in its ability to express proteins that can be incorporated into the basic protein processing machinery of the cell for use in modifying product proteins expressed later on. Budker *et al.* [32] have developed an improved gene transfer system for mammalian systems involving cationic liposomes. Also of interest is the work of Mattanovich *et al.* [33•] on the determination of the rate-limiting steps in gene expression in *E. coli* for two widely utilized expression systems.

Analytical tools

Currently, the major analytical tool for performing real-time analyses of metabolite flux and energy metabolism is NMR. One drawback of *in vivo* NMR is that high cell densities are required to obtain the necessary sensitivity. This creates problems when dealing with aerobic microorganisms because conventional reactor systems are incapable of supplying the required oxygen. Hartbrich *et al.* [34•] have developed a new reactor, the membrane cyclone reactor, which can be operated in chemostat mode and has high oxygen transfer capabilities, thus allowing *in vivo* NMR in aerobic systems. This new design was tested successfully with both an aerobe, *Corynebacterium glutamicum*, and an anaerobe, *Zymomonas mobilis*. Another way to get real-time information on metabolism is to add an easily measured marker gene to an organism. An application of this approach is the measurement of ATP levels in *E. coli* transformed with firefly luciferase [35•].

The bioluminescence was shown to correlate well with intracellular ATP levels during the lag and exponential growth phases.

A potentially powerful analytical technique for gaining information on metabolic fluxes is the use of biosynthetic fractional ^{13}C-labeling of amino acids coupled with two-dimensional NMR [36••]. This is not a real-time method; however, it can be used in conjunction with stoichiometric information to determine metabolic fluxes that are difficult to determine by other means.

Mathematical and computational tools

As the body of biological information grows, bioinformatics will become increasingly important. A recent issue of *Trends in Biotechnology* [37] was devoted to this field and provides several articles on current and future developments. Of special interest is a comprehensive listing of sources of bioinformation, including database resources, such as EcoCyc, which is a database that combines information about the genome and intermediary metabolism of *E. coli* [38]. More than 2000 genes and 300 enzymes encoded by those genes are detailed. In addition, 580 metabolic reactions are organized into 100 metabolic pathways.

A novel optimization strategy, based on a mixed-integer linear programming formulation, for maximizing the performance of a given metabolic network through the selection of optimal regulatory constraints and enzyme levels has been developed [39••]. This approach contrasts with stoichiometric treatments of metabolic systems, which do not contain any kinetic information, and cannot, therefore, be used to quantify the effect of changes in enzyme activities or regulatory constraints. It also differs from biochemical systems theory (BST) and metabolic control analysis (MCA), which only provide information about the effect on the metabolic network of changes in some external manipulated variable, such as enzyme activity, and consequently do not allow optimization of the regulatory superstructure.

Theoretical extensions of MCA allow the simplification of metabolic networks for modeling or experimental determination of control coefficients. Delgado and Liao [40•] provide justification for lumping together groups of reactions on the basis of their timescale separation, thus reducing the number of control coefficients that need to be determined. Rohwer *et al.* [41] indicate that it is possible to recognize monofunctional units or subsystems within a system. Subsystem metabolites are only produced or consumed within the subsystem, or by a limited number of fluxes crossing the subsystem boundary. The advantage of recognizing monofunctional units is that only one reaction within the unit need be perturbed during the determination of the control structure of the overall system. This results in a reduction in the number of control coefficients that need to be determined. Another theoretical development, which applies to systems with moiety conserved cycles, details how the global control properties of a system (flux and metabolite control coefficients in MCA or logarithmic gains in BST) can be expressed as a function of its local properties (elasticity coefficients or kinetic orders) or vice versa, through a single matrix inversion [42].

In addition to the various theoretical developments related to the quantitative characterization of metabolic systems, there have also been several examples of the practical application of both MCA and metabolic flux analysis (MFA) [43•–47•,48,49•]. Control coefficients were determined for the first four enzymes involved in glycolytic flux in *Z. mobilis* [43•]. Glucose-6-phosphate dehydrogenase was found to have the highest control coefficient (0.40), and overexpression of this enzyme increased glycolytic flux by 10–13%. A quantitative model describing penicillin synthesis in *Penicillium chrysogenum* has been developed [44•]. The model enabled calculation of the control and elasticity coefficients for the entire pathway and of the various fluxes associated with penicillin synthesis. In a separate study, the control coefficients for the first two enzymes involved in penicillin biosynthesis were determined [45•]. The initial step was found to be rate-limiting (control coefficient close to 1) during the early stages of a fed-batch production process, with control shifting to the second step in the later stages.

Vanrolleghem *et al.* [46•] provide an excellent example of validating a proposed metabolic network in *S. cerevisiae* through experimental analysis and MFA. Central metabolism in *C. glutamicum* was studied through a combination of MFA and ^{13}C NMR [47•]. In this instance, a comprehensive treatment of all the metabolic branch-points, rather than a portion of central metabolism, would also allow extension to other systems. Zupke and Stephanopoulos [48] developed a comprehensive approach for metabolic flux determination in hybridoma cells using ^{13}C NMR. Finally, Bonarius *et al.* [49•], in a study of metabolic fluxes in the central metabolism of hybridoma cells, show that the majority of glucose is utilized by the pentose phosphate pathway under most conditions, but especially in rapidly proliferating cells.

Conclusions, future directions and considerations

The field of metabolic engineering is now fairly well established and will advance rapidly in the future. As mentioned in the introduction, the availability of completely sequenced genomes will contribute significantly to this advance. Where it was once time consuming and costly to obtain new genes, in many cases it is now just a matter of searching on the Internet. The availability of sequenced organisms will also lead to a deeper understanding of cell physiology and metabolism. For example, Mushegian and Koonin [50••] have compared the genomes of *H. influenzae* and *M. genitalium* and concluded that only approximately

256 genes are necessary and sufficient for a modern-type cell. Such an analysis indicates that current industrial microorganisms may be more complicated than necessary for any particular industrial application. Koob *et al.* [51] have discussed the motivation for using simplified, or minimized, cells in biotechnology and have proposed a strategy, based on knowledge of the genome, for minimizing *E. coli*. The selection and customization of host organisms is already an important issue in metabolic engineering [7,18••], and will become more so in the 'post-genomic' era.

Metabolic engineering owes its initial successes to recombinant DNA technology, which has made possible the targeted modification of specific metabolic pathways and cell properties. As the field matures, however, hybrid approaches that involve both directed and evolutionary steps are likely to become increasingly important. For example, genetic engineering can first be used to add a gene or set of genes to an organism; an evolutionary approach, such as selection in a continuous reactor, can then be used to achieve further improvements in factors such as growth rate, regulatory properties or resistance to toxic metabolites. On a related topic, combinatorial methods will continue to be important in metabolic engineering, especially for drug discovery. Such methods have been particularly useful for the production of novel polyketides; a recent example is reported by Kao *et al.* [52].

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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2. Frost JW, Draths KM: **Biocatalytic syntheses of aromatics from D-glucose: renewable microbial sources of aromatic compounds.** *Annu Rev Microbiol* 1995, **49**:557–579.

3. Bailey JE: **Chemical engineering of cellular processes.** *Chem Eng Sc* 1995, **50**:4091–4108.

4. • Bailey JE, Sburlati A, Hatzimanikatis V, Lee K, Renner WA, Tsai PE: **Inverse metabolic engineering: a strategy for directed genetic engineering of useful phenotypes.** *Biotechnol Bioeng* 1996, **52**:109–121.

The authors put forth the concept of inverse metabolic engineering in contrast to 'constructive metabolic engineering'. Inverse metabolic engineering is described as the process of identifying a desired phenotype, determining the basis for this phenotype, and then transferring the gene(s) for this phenotype into the desired host organism. Several examples from the authors' laboratory are described, including the use of VHb to alleviate oxygen limitation and the expression of cyclin E in CHO cells to eliminate the need for exogenous mitogens in cell culture.

5. • Liao JC, Hou SY, Chao YP: **Pathway analysis, engineering and physiological considerations for redirecting central metabolism.** *Biotechnol Bioeng* 1996, **52**:129–140.

This article reviews some of the recent work in the authors' laboratory on flux analysis, the PTS, the production of intermediates for the common aromatic pathway and aspects of global regulation. It also extends some of this work and describes the mathematical analysis of the production of DAHP, the first metabolite in the common aromatic pathway, in detail.

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This paper is the first report of the complete genome sequence of a free-living organism.

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This paper is the first report of the complete genome sequence of an archaeon.

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This paper reports the first complete genome sequence of a eukaryotic organism, *S. cerevisiae*.

13. • Millard CS, Chao YP, Liao JC, Donnelly MI: **Enhanced production of succinic acid by overexpression of phosphoenolpyruvate carboxylase in *Escherichia coli*.** *Appl Environ Microbiol* 1996, **62**:1808–1810.

PEP occupies a critical position in metabolism and thus has been the subject of much metabolic engineering activity. This paper compares the influence of the overexpression of two enzymes, PEP carboxylase and PEP carboxykinase, on the production of succinic acid by *E. coli*. Overexpression of PEP carboxylase caused an increase in succinic acid production from glucose whereas PEP carboxykinase did not.

14. • Porro D, Brambilla L, Ranzi BM, Martegani E, Alberghina L: **Development of metabolically engineered *Saccharomyces cerevisiae* cells for the production of lactic acid.** *Biotechnol Prog* 1995, **11**:294–298.

A bovine lactate dehydrogenase gene was expressed in *S. cerevisiae* and the recombinant strain was shown to produce lactic acid. In an attempt to improve the production of lactic acid, modified constitutive and inducible promoter sequences were tested, but their use did not lead to improved lactic acid production. Improved production was finally obtained using a two-stage batch cultivation process. This paper illustrates the importance of utilizing a combined metabolic engineering/bioprocessing engineering approach for process improvement.

15. • Platteeuw C, Hugenholtz J, Starrenburg M, Van Alen-Boerrigter I, De Vos WM: **Metabolic engineering of *Lactococcus lactis*: influence of the overproduction of α-acetolactate synthase in strains deficient in lactate dehydrogenase as a function of culture condition.** *Appl Environ Microbiol* 1995, **61**:3967–3971.

L. lactis converts sugars to a variety of products, including diacetyl, acetoin, and 2,3-butanediol. This paper demonstrates the alteration of the product mix by both altering the fermentation conditions and modulating the expression of key enzymes in the pathway. It is also an example of a pathway where both enzymatic and nonenzymatic activities are significant.

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18. Flores N, Xiao J, Berry A, Bolivar F, Valle F: **Pathway engineering**
•• **for the production of aromatic compounds in *Escherichia coli*.** *Nat Biotechnol* 1996, **14**:620–623.

PEP is an important intermediate in the production of aromatic amino acids and related compounds. In *E. coli*, glucose is transported by the PTS that is stoichiometrically coupled to the use of PEP. In this paper, the authors describe the use of chemostat culture to select strains of *E. coli* that transfer glucose without the use of PEP. They then demonstrate the utility of these strains for the production of the aromatic precursor DAHP. This paper illustrates the importance of selection and 'evolutionary' approaches in metabolic engineering.

19. Gosset G, Yong-Xiao J, Berry A: **A direct comparison**
• **of approaches for increasing carbon flow to aromatic biosynthesis in *Escherichia coli*.** *J Indust Microbiol* 1996, **17**:47–52.

This paper extends the work described in [18••] by combining several approaches to improve PEP management. A strain containing all of the modifications showed nearly a 20-fold increase in carbon commitment to aromatic biosynthesis.

20. John ME, Keller G: **Metabolic pathway engineering in cotton:**
•• **biosynthesis of polyhydroxybutyrate in fiber cells.** *Proc Natl Acad Sci USA* 1996, **93**:12768–12773.

This paper is significant for several reasons. First, it describes the use of metabolic engineering for the production of PHB, a commercially important polymer. Second, it demonstrates the production of this polymer in a plant. The modification of agricultural crops to produce value-added chemicals is of great current interest. Third, it illustrates the use of metabolic engineering to produce a complex composite material.

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22. Suyama A, Iwakiri R, Kimura N, Nishi A, Nakamura K, Furukawa K: **Engineering hybrid pseudomonads capable of utilizing a wide range of aromatic hydrocarbons and of efficient degradation of trichloroethylene.** *J Bacteriol* 1996, **178**:4039–4046.

23. Tsai PS, Hatzimanikatis V, Bailey JE: **Effect of *Vitreoscilla***
• **hemoglobin dosage on microaerobic *Escherichia coli* carbon and energy metabolism.** *Biotechnol Bioeng* 1996, **49**:139–150.

Much work has been done on the use of VHb to improve the performance of organisms under conditions of suboptimal aeration. This paper provides a detailed analysis of metabolic flux in *E. coli* expressing various levels of VHb. The flux analysis indicates that VHb-expressing cells direct a greater fraction of their metabolic flux toward the pentose phosphate pathway than nonexpressing cells.

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This paper explores the mechanism by which VHb enhances the performance of *E. coli* under conditions of suboptimal aeration. Apparently, the reason for the improvement is that VHb somehow increases the level and activity of terminal oxidases.

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27. Erbe JL, Taylor KB, Hall LM: **Expression of mouse metallothionein in the cyanobacterium *Synechococcus* PCC7942.** *J Indust Microbiol* 1996, **17**:41–46.

28. Chaplen FWR, Fahl WE, Cameron DC: **Effect of endogenous**
• **methylglyoxal on Chinese hamster ovary cells grown in culture.** *Cytotechnology* 1996, **22**:33–42.

Methylglyoxal is a toxic metabolite that is produced at low levels in nearly all cells. The major mechanism for removal of methylglyoxal is the glyoxalase system, which converts methylglyoxal to D-lactic acid. The first enzyme in this system is glyoxalase I. In this paper, it was shown that under certain growth conditions, CHO cells have increased intracellular levels of methylglyoxal. It was then shown that these levels can be reduced, and cell survival can be improved, by the overexpression of *P. putida* glyoxalase I activity in CHO cells.

29. Wang X, Da Silva NA: **Site-specific integration of heterologous**
•• **genes in yeast via Ty3 retrotransposon.** *Biotechnol Bioeng* 1996, **51**:703–712.

The stable expression of gene products is necessary for all recombinant cell-based processes. This study describes the development and optimization of a system for chromosomally inserting and maintaining multiple, stable gene copies in yeast. With the Ty3 retrotransposon system, these insertions occur at nonessential sites in the chromosome. By varying the integration protocol, it was possible to achieve one, two, three or four gene integrations with each round of amplification. Subsequent rounds of integration could be used to insert further copies of the original gene or copies of different genes. This ability to employ the system repeatedly and for different genes will confer considerable flexibility to the metabolic engineering of yeast.

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C. thermosaccharolyticum was recently renamed *Thermoanaerobacterium thermosaccharolyticum* by the American Type Culture Collection. This paper describes the transformation of this potentially industrially useful chemical producer with pCTC1, a shuttle vector based the broad host range Gram-positive bacterial replicon pAMβ1 and the Gram-negative bacterial R2 bacterial replicon. Transformation efficiencies were low.

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This work describes the development of a new type of baculovirus expression vector that can express heterologous proteins immediately following infection using the viral ie1 promoter. This type of vector was used for protein production during the early stages of infection, thus allowing gene products to be incorporated into the basic protein processing machinery of the insect cell for use in modifying proteins expressed later in infection.

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This paper evaluates whether transcription or translation is the rate-limiting step for two widely utilized gene expression systems, one employing the phage T7 RNA polymerase, the other employing the *tac* promoter and associated ribosomal binding site. For the phage T7 system, transcription was not rate limiting, because this system is highly efficient; however, for the *tac* promoter system, transcription was rate limiting, mainly because of poor ribosomal binding at the native ribosomal binding site. Mutation of the *tac*-associated ribosomal binding site for closer homology with a more effective site resulted in increased translation and, less obviously, in increased transcription.

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This paper describes the development of a new reactor design that allows ^{13}C and ^{31}P NMR studies of aerobic microbial cultures. The membrane cyclone reactor can be operated in chemostat mode and has very high oxygen-transfer capabilities, which permitted the growth of dense cultures within the NMR measurement chamber. Although the effective sensitivity of this system was lower than some previously reported designs, it had the advantage of allowing reproducible *in vivo* measurements of flux distribution in microorganisms under well-defined physiological conditions.

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• **concentrations in *Escherichia coli* fermentations.** *Biotechnol Bioeng* 1996, **52**:364–372.

This paper describes an approach utilizing firefly luciferase for the continuous monitoring of ATP levels present in cells grown in aerobic cultures. The luciferase cDNA was plasmid borne and driven by a constitutive promoter. Reliable readings of intracellular ATP levels could be taken during lag and exponential phase growth providing dissolved oxygen was maintained above 15% of air saturation.

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•• **of proteinogenic amino acids: an efficient analytical tool to**

investigate intermediary metabolism. *Eur J Biochem* 1995, **232**:433–448.
It is typically not possible to get enough information to completely determine intracellular fluxes from rates of substrate consumption, product formation and cell growth. This paper describes a method that can potentially provide the additional information needed to fully determine such fluxes. It is based on growing cells on a mixture of unlabelled and completely ^{13}C-labelled glucose. Depending on the metabolic flux, various amino acid precursors are labelled differently. These differences are 'recorded' in the amino acid residues of proteins. The differences can later be 'read' using two-dimensional ^{13}C NMR.

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•• **of metabolic reaction networks via mixed-integer linear optimization.** *AIChE J* 1996, **42**:1277–1292.
This paper describes a novel optimization strategy that allows the selection of the optimal regulatory structure for a particular metabolic process. This strategy utilizes a mixed-integer linear programming formulation to identify the changes in a given regulatory network necessary to achieve the desired objective. As an example, the optimal regulatory structure for the maximization of phenylalanine production is presented. Inactivation of three of the eight original feedback inhibition loops and overexpression of three enzymes would theoretically cause a 42% increase in phenylalanine selectivity.

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Systems for which the eigenvalues of the Jacobian of the system are widely separate exhibit time-scale separation. Utilizing this property of the system, it is shown that time-scale separation is an effective way of localizing the control of a metabolic pathway to a few enzymes. 'Fast' reactions are characterized as those for which the enzymes are consistently overexpressed by an organism, as opposed to 'slow' reactions which are genetically regulated to control the flux through the pathway. The control coefficients for 'fast' reactions are small relative to those for 'slow' reactions, and this represents justification for lumping the 'fast' reactions together in order to reduce the number of control coefficients that need to be determined.

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