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METABOLIC ENGINEERING

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■ **Abstract** Metabolic engineering is the science that combines systematic analysis of metabolic and other pathways with molecular biological techniques to improve cellular properties by designing and implementing rational genetic modifications. As such, metabolic engineering deals with the measurement of metabolic fluxes and elucidation of their control as determinants of metabolic function and cell physiology. A novel aspect of metabolic engineering is that it departs from the traditional reductionist paradigm of cellular metabolism, taking instead a holistic view. In this sense, metabolic engineering is well suited as a framework for the analysis of genome-wide differential gene expression data, in combination with data on protein content and in vivo metabolic fluxes. The insights of the integrated view of metabolism generated by metabolic engineering will have profound implications in biotechnological applications, as well as in devising rational strategies for target selection for screening candidate drugs or designing gene therapies. In this article we review basic concepts of metabolic engineering and provide examples of applications in the production of primary and secondary metabolites, improving cellular properties, and biomedical engineering.

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INTRODUCTION

Altering metabolic pathways to improve cell properties and the chances of cell survival is as old as nature itself. The genomic and metabolic evolution of extremophiles is an example of such a natural adaptation process in bacteria (96). The

intentional manipulation of metabolic pathways by humans to improve the properties and productivity of microorganisms is similarly an established concept. Techniques such as genetic modifications via random mutagenesis have yielded, for example, improved strains of *Corynebacterium glutamicum* and its related species *Brevibacterium lactofermentum* and *Brevibacterium flavum* that excrete large amounts of amino acids into the fermentation medium (52, 80, 106). Random mutagenesis relies heavily on chemical mutagens and creative selection techniques to identify superior strains for achieving a certain objective. Such traditional genetic approaches for strain improvement have been applied extensively in the past also in the areas of antibiotics, solvents, and vitamin production among others.

Since the past decade, the development of recombinant DNA techniques has introduced a new dimension to pathway manipulation by offering, for the first time, the capability to construct specific metabolic configurations with novel, beneficial characteristics. Genetic engineering allows precise modification of specific enzymatic reactions in metabolic pathways, leading to the construction of well-defined genetic backgrounds. The redirection of cellular metabolism to create or enhance desirable attributes has been accomplished with a variety of novel techniques and applied towards an even greater variety of goals.

In this context, metabolic engineering has emerged as the technological and scientific discipline dealing with the introduction of specific modifications to metabolic pathways to improve cellular properties. Metabolic engineering involves manipulation of enzymatic, transport, and regulatory functions of the cell by using recombinant DNA technology (5, 86). First, various analytical techniques are used to identify and subsequently determine fluxes through critical metabolic pathways in the cell or tissue of interest. This knowledge provides the rational basis for applying, in the second step, molecular biological techniques to enhance metabolic flux through a pathway of interest and minimize metabolic flow to undesired biosynthetically related products. Although a certain sense of direction is inherent in all strain improvement programs, the directionality of effort is a strong focal point of metabolic engineering, compared with random mutagenesis, because this directionality plays a dominant role in enzymatic target selection, experimental design, and data analysis.

Although various terms have been coined over the past two decades to represent the increasing activity in pathway modification (pathway engineering, cellular engineering, in vitro evolution, etc) (58, 65), the term metabolic engineering has succeeded in capturing the ever growing interest in this area. Furthermore, although initially embodied as a collection of examples from the chemical industry and biomedical research, metabolic engineering is quickly becoming a distinct scientific field. Its novel contribution lies in its emphasis on complete metabolic networks rather than individual reactions. To elaborate, as with all traditional fields of engineering, metabolic engineering too encompasses the two defining steps of analysis and synthesis. Because metabolic engineering emerged with DNA recombination as the enabling technology, its initial focus was on synthesis

in the form of new pathway construction. As such, differentiation from genetic engineering was initially diffuse, and metabolic engineering could be considered as the technological manifestation of applied molecular biology. The real contribution of metabolic engineering emerged as soon as a need for a more rational approach to identifying promising targets of metabolic manipulation was articulated, replacing the previous, mostly ad hoc target selection process. In this sense the contribution of metabolic engineering emanates from pathway analysis, which yields an enhanced perspective on metabolism and cellular function, including consideration of reactions in their entirety rather than in isolation. Thus, metabolic engineering seeks to analyze and then synthesize and design, using techniques and information developed from extensive reductionist research.

Metabolic engineering has found many applications, especially in microbial fermentation. It has been applied to increase the production of chemicals that are already produced by the host organism (e.g. 13, 15, 22, 34, 92), to produce desired chemical substances from less expensive feedstocks (e.g. 6, 53, 107), and to generate products that are new to the host organism (e.g. 67, 68). Other challenges associated with metabolic engineering are the biosynthesis of secondary metabolites, the generation of organisms with desirable growth characteristics, and the manipulation of pathways for the production of chiral compounds as intermediates in the synthesis of pharmaceutical products (38). Finally, although less widely appreciated, metabolic engineering techniques can also be applied for studying physiological systems and isolated whole organs in vivo to elucidate the metabolic patterns that occur in different physiological states, such as ''fed'' or ''fasted,'' as well as in disease (105).

METABOLIC FLUXES

Because metabolic pathways and fluxes are at the core of metabolic engineering, it is important to elaborate on their definition. A metabolic pathway is defined as any sequence of feasible and observable biochemical-reaction steps connecting a specified set of input and output metabolites. The pathway flux is the rate at which input metabolites are processed to form output metabolites (84). The importance of feasibility and observability should be noted in the definition, in view of the diversity and complexity of various metabolic maps. If some metabolic fluxes in a pathway or metabolic network cannot be determined independently, it is better to lump these reactions together, because their inclusion provides no additional information.

The determination of intracellular fluxes, along with analysis of factors affecting flux distributions, is collectively referred to as metabolic flux analysis (MFA). MFA combines data on uptake and secretion rates, biosynthetic requirements, metabolic stoichiometry, and quasi–steady-state mass balances on metabolic intermediates to determine intracellular metabolic fluxes (84). MFA has been the focus of attention of many researchers in the past and has yielded important

information on flux distribution and its control in many bacteria. The combination of analytical methods to quantify fluxes and their control with molecular biological techniques to implement suggested genetic modifications is the essence of metabolic engineering. An iterative cycle of genetic change followed by an analysis of its consequences and design of further modifications, analogous to that articulated for protein engineering, can also be applied in the development of an optimized strain (3). The flux thus becomes a focal point of metabolic engineering and justifies further research for the development of methods for its determination in vivo.

MFA also reveals the degree of pathway engagement in the overall metabolic process. Furthermore, elucidation of the control of flux provides a mechanistic basis for rationalizing observed fluxes and flux distributions at key metabolic branch points (86). As these fluxes are determined under in vivo conditions, MFA also allows valid comparisons to be made between in vivo and in vitro enzymatic behavior. Finally, the flux is a fundamental determinant of cell physiology and a critical parameter to use when comparing the behavior of strain variants. Even if the fermentation characteristics of such strains differ, such differences may be relatively unimportant if the flux distributions around key branch points have not been altered.

Intracellular fluxes are calculated by MFA with a stoichiometric model for the internal reactions and mass balances around intracellular metabolites (98). Required data are obtained from measured extracellular fluxes, such as substrate uptake rates and metabolite secretion rates (85). Intracellular fluxes from reactions that are cyclical or that split and later converge may be determined by assaying asymmetries in the distribution of radio-labeled atoms of intermediate metabolites (77, 83). The metabolic fluxes determined by MFA provide a comprehensive perspective of the control system at work in metabolic networks. This control system can be described in terms of metabolic control coefficients (30, 41). Flux control coefficients are measures of the degree of control exercised by an enzyme on the overall network flux and can be determined by measuring flux responses to metabolic perturbations.

As mentioned in the previous paragraphs, elucidation of the flux control structure of metabolic pathways offers tremendous opportunities for the rational design of the optimal genotype of a cellular catalyst. This activity should be viewed as complementary to molecular biological toolboxes for implementing gene transfers and other similar modifications. In fact, recombinant technology has advanced so rapidly in recent years that rational analysis of metabolic pathways for the identification of target genes and enzymes is the limiting component in the directed optimization of cellular function. Evidence for this assertion is the observation that currently, almost 20 years after the pioneering developments in genetic engineering, we have hardly begun to harness the potential of modern biotechnology in the areas of fuels, chemicals, or materials production.

In the following sections, we review a few illustrative applications of metabolic-pathway manipulation. We organize the various applications of metabolic

engineering into four basic groups: (*a*) improving primary metabolite production, (*b*) improving secondary metabolite and biopharmaceutical production, (*c*) improving cell properties, and (*d*) improving the biomedical field. We have three goals in reviewing these applications: first, to provide a sample of the truly broad range of possibilities for biocatalyst improvement afforded by pathway manipulation and metabolic engineering; second, to alert the reader to the complexity of metabolic pathways, along with their regulation and need of coordination with overall metabolism; and, third, to underscore the methods used for effecting desired changes in cellular systems for industrial use or medical reasons. This review concludes with some ideas and suggestions for future directions of the field.

PRIMARY METABOLITES

A large number of mainly industrial applications can be classified as primary metabolites. A central goal of metabolic engineering is to achieve the production of commodity chemicals by overexpressing key metabolic pathways that already exist in the host organism or by introducing new routes of metabolism. We next review efforts in metabolic engineering to improve the yield and productivity of ethanol, amino acids, and solvents.

A major challenge of using biotechnology in the industrial production of fuels has long been the construction of microorganisms that are able to ferment inexpensive and abundant carbon resources, such as lignocellulosic materials, into ethanol for use as a biofuel, among other applications. The most commonly used ethanol producer, *Saccharomyces cerevisiae,* cannot ferment pentoses, which may constitute 8%–28% of lignocellulose. On the other hand, other bacteria such as *Erwinia chrysanthemi, Klebsiella planticola,* and *Escherichia coli* can grow efficiently on a wide range of carbon substrates that includes five-carbon sugars, but competing pathways divert carbon flow away from ethanol production. Initial studies were only partially successful in redirecting fermentative metabolism to ethanol production in these bacteria, by amplifying their pyruvate decarboxylase activity (7, 91, 92). A further improvement of the process was achieved by cloning and overexpressing the *Zymomonas mobilis adhB* gene, yielding recombinants of *E coli* (34) and *Klebsiella oxytoca* (67, 68, 104) that efficiently ferment a variety of sugars to ethanol. Ohta et al investigated the expression of the pyruvate decarboxylase and alcohol dehydrogenase genes of *Z. mobilis* in a related enteric bacterium, *K. oxytoca* (67, 68). *Klebsiella* strains have two additional fermentation pathways not present in *E. coli,* which are used to convert pyruvate to succinate and butanediol. As for *E. coli,* it was possible to divert $>$90% of the carbon flow from sugar catabolism away from the native fermentative pathways and toward ethanol.

Amino acid production is also a heavily researched area. Tryptophan synthesis in *E. coli* is highly regulated by a complex set of feedback mechanisms. By

transducing each of several mutations one at a time, researchers combined, within a single strain, a long list of alterations to these mechanisms, thus creating a tryptophan overproducer (1, 82). A *Corynebacterium glutamicum* strain able to produce 18 g liter^{-1} of tryptophan has been altered to produce large amounts of tyrosine (26 g liter^{-1}) by overexpressing deregulated 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase and chorismate mutase (33). Overexpression of an additional gene, prephenate dehydratase, in the previous construct led to the predominant production of phenylalanine (28 g liter^{-1}). In a similar way, significant progress was made recently in efforts to construct efficient threonine-producing strains by metabolic engineering. The genes that are involved in the threonine production pathway of *C. glutamicum* were cloned, and the regulatory properties of the enzymes encoded by them were well characterized (e.g. 20, 22, 39). The cloning of a deregulated (threonine-insensitive) homoserine dehydrogenase, along with modulation of the activity of homoserine kinase relative to that of homoserine dehydrogenase (the first two enzymes in the threonine pathway), yielded threonine-secreting strains (14). Similarly, overexpression of *ilvA*, the first gene in the isoleucine pathway, allowed significant isoleucine accumulation by *B. lactofermentum* strains (15).

Another industrially significant primary metabolite is 1,3-propanediol (1,3-PD), an intermediate in chemical and polymer synthesis, for example, in the synthesis of polyurethanes and polyesters. 1,3-PD is currently derived from petroleum, and it is expensive to produce relative to similar diols. Tong & Cameron (93) constructed an *E. coli* propanediol-producing strain carrying genes from the *Klebsiella pneumoniae* dihydroxyacetone *(dha)* regulon. These genes allow the strain to grow anaerobically on glycerol and produce 1,3-PD. A further process improvement via metabolic engineering in the same field is in production of 1,3-PD by using sugars as a carbon source, because sugars are significantly less expensive than glycerol. No known natural organism ferments sugars directly to 1,3-PD. One way to replace, at least partially, the need for glycerol is by cofermentation of glycerol and a sugar such as glucose. Cofermentation is not possible with native 1,3-PD producers because glucose represses the 1,3-PD pathway. *E. coli* that has been transformed with the *K. pneumoniae* 1,3-PD oxidoreductase gene (*dhaT)* and the glycerol dehydratase gene *(dhaB)* of the same organism is able to coferment glycerol and glucose (93, 94). The glycerol is converted primarily to 1,3-PD, and the glucose is used for growth and regeneration of reducing potential. Until today the production of 1,3-PD by fermenting sugars alone has not been possible to a commercially advantageous extent. The initial success with the metabolic engineering of 1,3-PD production, coupled with the complexity and difficulty of rational process improvement with *Thermoanaerobacterium thermosaccharolyticum*, the best naturally occurring organism for the fermentation of sugars to 1,2-propanediol, led to the construction of pathways in *E. coli* similar to those used by *T. thermosaccharolyticum.* This was accomplished by overexpressing the native glycerol dehydrogenase of *E. coli* or cloning the aldolase reductase gene from rat lens cDNA into *E. coli.* Either of these two strains led to

the production of 1,2-PD in equal amounts in batch and continuous culture. These results provide the first example of recombinant organisms able to ferment sugars to 1,2-PD and demonstrate that various strategies for the fermentation of sugars to 1,3-PD are possible (9).

Another example of converting native metabolic intermediates to desirable end products is the production of the β-carotene precursor to vitamin A. By introducing the three carotenogenic genes required for lycopene synthesis from farenyl diphosphate under the control of *Candida utilis*, a *C. utilis* strain producing 1.1 mg of lycopene/g (dry weight) of cells has been generated. By using concepts of metabolic engineering with *C. utilis*, carbon flux in this yeast strain was redirected away from ergosterol formation for potential use by the carotenoid pathway. The influential steps in the pathway that were manipulated were 3-hydroxy methylglutaryl (HMG)-coenzyme A (CoA) reductase, encoded by the *HMG* gene, and squalene synthase, encoded by the *ERG9* gene. A combination of *ERG9* gene disruption and the overexpression of the *HMG* catalytic domain gave the highest lycopene yield. These findings illustrate how modifications in related biochemical pathways can be used to enhance the production of commercially desirable compounds such as carotenoids (81).

Scientists are now beginning to elucidate the pathway of vitamin C biosynthesis in higher plants. Several enzymes and precursors at work in this pathway have recently been identified (103), and MFA may in the near future allow for their rational modification. To this end, Sauer et al (76) investigated fluxes in a riboflavin-producing *Bacillus subtilis* strain and found that generation of the desired riboflavin was limited by the biosynthetic pathway fluxes and not by the fluxes associated with central carbon metabolism.

Another group of metabolic engineering applications is the engineering of organisms that can use abundant byproducts of various industries as nutrients by extending the range of their substrates. Whey, with high lactose and protein content, is a nutrient-rich byproduct of the dairy industry that can provide an inexpensive carbon and nitrogen source in biotechnological processes. Although a variety of microbes can use whey, some of the most industrially prominent organisms are unable to do so. The *E. coli lacZY* operon (coding for β-galactosidase and lactose permease) was inserted into *Xanthomonas campestris*, a bacterium that is used for xanthan gum production (24). The recombinant stain expressed high levels of β-galactosidase and grew well in a medium containing lactose as the sole carbon source. In another approach, an *S. cerevisiae* recombinant strain was constructed that expressed the gene for a secreted β-galactosidase (*lacA*) from *Aspergillus niger* (53). This approach offers significant advantages over earlier processes for the fermentation of whey by *S. cerevisiae*, which used either β-galactosidase–prehydrolyzed whey or yeast coimmobilized with β-galactosidase. Sucrose is another abundant and inexpensive carbon source found in, for example, cane molasses. A successful attempt to create a recombinant sucrose-metabolizing strain involved the cloning of the *scrA* gene, which codes for sucrase, from *E. coli* B-62 onto a plasmid and then transferring the cloned DNA fragment onto

the chromosome of *E. coli* K-12. Tryptophan producer derivatives of *E. coli* K-12 expressing the *scrA* gene grew well in sucrose medium and excreted amounts of tryptophan comparable to these from similar strains grown on glucose (95). Starch, derived from renewable sources such as corn and cereals, is a very important carbon and energy source in biotechnological processes. It is a mixture of linear and branched homopolymers of D-glucose. Because most microorganisms are unable to degrade this glucose biopolymer, work has focused on cloning genes for enzymatic starch hydrolysis into various organisms (49). Along these lines, an *S. cerevisiae* strain was constructed that contained a glucoamylase gene from *Aspergillus* sp. (35). The recombinant strain was able to grow on amylodextrins, albeit at a lower rate than occurs when glucoamylases are added to the fermentation medium.

Cofactor engineering is a novel approach to metabolic engineering. The cloning of the *Streptococcus* mutant *nox-2* gene, coding for the H_2O-forming (nontoxic) NADH oxidase, under the control of the *nisA* promoter in *Lactococcus lactis*, provides a powerful tool with which to attempt the modulation of the metabolism. The main effect of overproducing the NADH oxidase was an observed decrease in the NADH/NAD ratio under aerobic conditions. This engineered system could be used to provoke a shift from homolactic to mixed-acid fermentation during aerobic glucose catabolism. The magnitude of this shift was directly dependent on the level of NADH oxidase overproduced. These results indicate that the observed shift from homolactic to mixed-acid fermentation under aerobic conditions is mainly modulated by the level of NADH oxidation resulting in low NADH/NAD ratios in the cells (57).

SECONDARY METABOLITES

The challenges associated with engineering the biosynthesis of secondary metabolites are qualitatively different from those associated with the production of commodity bulk chemicals. Products of interest are often complex molecules that are necessarily derived from biological sources. In this arena, pathway engineering may increase the efficiency of existing production methods but may also lead to the development of new products. The relatively high value of these products shifts the emphasis from economics and efficiency to innovation.

An interesting example is the production of antibiotics. Antibiotics are made by secondary metabolic pathways that use common metabolites in less specific and, sometimes, more intricate ways than metabolites are used in primary metabolism. Recently, it has become apparent that yields of secondary metabolites, including antibiotics, can also be improved by overcoming rate-controlling biosynthetic steps through genetic techniques. In addition, metabolic engineering techniques are applied to modify known antibiotics to improve their properties and also to synthesize new product forms.

Among various antibiotic producers of industrial importance, *Streptomyces* species rank near the top. Actinorhodin biosynthesis genes were transferred from

S. coelicitor, the only species with well-established genetics, to *S. lividans*, enabling the latter strain to produce actinorhodin. Later, clustered erythromycin genes from *S. erythreus* were transferred to *S. lividans*, allowing the recombinant strain to produce erythromycin A. Transformation of the fungi *Neurospora crassa* and *A niger* with a cosmid containing *Penicillium chrysogenum* penicillin biosynthetic genes resulted in the production of penicillin V by these strains (4, 60).

The manufacture of antitumor drugs, many of which are natural products, has also received significant attention. The undesirable side effects of antitumor drugs, as well as the development of resistance to them, have fueled the need for the discovery of novel therapeutic agents and the means for their synthesis. The manipulation at a genetic level of the enzymes composing drug production pathways within cells has emerged as a very powerful tool for achieving these goals. This method, which has been termed combinatorial biosynthesis, has yielded derivatives and analogs of drugs such as mithramycin (21, 28), tylosin (2), erythromycin (19, 36, 37, 63, 99), and methymycin (108). Here, as an example, we focus on techniques used to engineer a biological reaction pathway for the production of the cancer chemotherapy drugs epirubicin (4′-epidoxorubicin) and 4′-epidaunorubicin. The medical and industrial significance of epirubicin lies in its antitumor activity, its use as a precursor of the drug candidate 4-iodoxorubicin (87), and its reduced cardiotoxicity relative to that of doxorubicin (102), a heavily prescribed chemotherapy medication. The currently used synthetic means of producing epirubicin suffers from many complexities. The scheme involves seven different synthesis steps, which are followed by still more separation and deprotection steps. Madduri et al have described an alternative process that generates 4′-epidaunorubicin and epirubicin directly and relies on the fermentation of a *Streptomyces peucetius* strain to which has been introduced an avermectin or erythromycin biosynthetic gene (59).

This discovery was made with the use of a *S. peucetius* strain in which the *dnmV* gene has been disrupted (69). This strain accumulates ε-rhodomycinone, a precursor of 4′-epidaunorubicin and epirubicin, unless transformed with a plasmid containing the wild-type *dnmV* gene. In this case, daunorubicin and doxorubicin, analogs of 4′-epidaunorubicin and epirubicin, respectively, are produced (97). The function of the DnmV enzyme was hypothesized to be that of a TDP-4-ketohexulose-reductase (26), much like that of the *Saccharopolyspora erythraea eryBIV* (89) and *Streptomyces avermitilis avrE* (66) gene products. Cloning of these latter two genes onto plasmids used to transform the *dnmV* mutant yielded bacteria that produced 4′-epidaunorubicin and epirubicin. The integration of the *avrE* gene directly into the *S. peucetius* chromosome gave results similar to those seen with the gene acting in *trans* from a plasmid.

Madduri et al also found that the yield of 4′-epidaunorubicin was increased by introducing a *dnrH* mutation into the *avrE* integrant strain (59). It is believed that the *dnrH* genes code for enzymes that catalyze side reactions that form glycosides of daunorubicin and its precursors (78). Additional productivity gains were achieved by providing an overexpressed plasmid copy of the *dnmT* gene into the *avrE* integrant. The enzyme DnmT is hypothesized to catalyze a limiting

step in the synthesis of the daunorubin precursor, daunosamine. By incorporating all three findings—the integration of *avrE*, the mutation of *dnrH*, and the overexpression of *dnmT*—into one strain, 4′-epidaunorubicin titers were realized that approximated those of daunorubicin seen in the wild-type strain. Although these product concentrations are still too low to effectively compete with the extant synthetic process results, the gains made by metabolic engineering are significant and point the way for future improvements that can be implemented once the entire biosynthetic network has been better characterized. Extensive reviews of combinatorial biosynthesis advances can be found elsewhere (32, 74).

Biological processes using reactions catalyzed by enantiospecific enzymes are increasingly being investigated as methods for the manufacture of pharmaceutical compounds and their intermediates. Because these compounds are often active, as a treatment, in only one particular chiral form, processes that exclusively generate the desired chiral form are very advantageous. Although biologically catalyzed reactions, termed biotransformations, do possess this attribute, their productivity is often economically unfavorable relative to yields seen in organic synthesis processes. Because of this, metabolic engineering is being called on to provide the means of increasing the efficiencies and product concentrations of the pathways of interest, so that they can be more attractive for use in industrial practice.

One potential application of biotransformations is the manufacture of Crixivan (indinavir sulfate), a protease inhibitor developed and produced by Merck & Co. and targeted for the treatment of human immunodeficiency virus (23). The chemical structure of Crixivan contains five chiral centers, two of which are contributed to the final compound from the intermediate 1-amino-2-(*R*)-indanol, a derivative of indene. This drug precursor can be produced by transformation of indene by the *Rhodococcus* sp. strain I24 (8). This soil isolate has been shown to grow with naphthalene and toluene as its only carbon sources, and it is believed to possess at least three different oxygenases. The presence of the multiple-oxygenase enzymes results in formation of multiple stereochemical enantiomers of the desired 1-amino-2-indanol when indene is supplied to I24 cultures. To increase both the specificity with which I24 produces the 2-(*R*) form and the final product titers, the pathway of indene bioconversion has been the subject of recent study. In particular, the nature of the specific oxygenases in action is being investigated, and factors controlling flux distribution to the different competing pathways analyzed. These studies are aided by novel methods of flux determination that make use of radio-labeled tracer compounds.

The polyketide family is another rich source of bioactive molecules with antibiotic and pharmacological properties. Reasons that polyketides are an attractive study model for metabolic engineering include the following: (*a*) their complex structure results from simple units combined in diverse ways; (*b*) the modular construction of the enzymatic catalyst allows control of enzyme structure and, hence, polyketide type at the genetic level. Recent progress in this area has established the groundwork to generate novel polyketide structures through genetic

engineering of polyketide synthases and at the same time to derive knowledge that elucidates the structure-function relationship in polyketide synthases (48, 64). Moreover the field provides an opportunity to bridge the fields of genetics and chemistry and, above all, promises to enable scientists to rationally design novel molecules at the level of DNA.

CELL AND TISSUE ENGINEERING

Metabolic engineering can also be used to construct cells with desirable properties by altering characteristics such as growth, proliferation, tolerance to exogenous factors, substrate utilization, etc. These characteristics are the result of complex biological functions involving multiple gene products. This complexity may afford greater efficiency or higher quality control or it may exist simply because a single protein cannot provide the required function. Metabolic engineering strategies that coordinately modify multigene expression therefore have the potential to achieve previously inaccessible metabolic states.

The hemoglobin (VHb) of the microorganism *Vitreoscilla* species has been extensively investigated in recent years as a tool for maintaining metabolic activity under hypoxic conditions. In its native host, VHb binds oxygen at a low extracellular concentration and acts as a buffering agent at high intracellular-oxygen concentrations, allowing the bacteria to survive in the hypoxic environments to which it is indigenous (100). The gene (*vgb*) encoding the hemoglobin was cloned (18, 51) and used to transform a wide range of species in attempts to improve respiration, growth, and productivity. A few of the most recent advances have involved increasing the total protein secretion, neutral protease, and α-amylase activities of *B. subtilis* (46), boosting antibiotic production in *S. coelicolor* (16) and *S. lividans* (60), enhancing lysine production in *C. glutamicum* (75), and improving the degradation of benzoic acid by *Xanthomonas maltophilia* (56). The effectiveness of VHb in improving cellular processes was compared with that of two other globins, horse heart myoglobin (HMb) and yeast flavohemoglobin (YFb), and found to be the superior growth enhancer in *E. coli* (47). The investigators theorized that this was so because, of the three globins, VHb was unique in possessing a slow oxygen on-rate constant and a fast oxygen off-rate constant. This results in VHb being particularly effective in both scavenging sparse oxygen molecules and donating them to the respiratory components that require oxygen. Another recent work has identified a strong effect of growth medium on the efficacy of improving processes with VHb: Wei et al (101) attempted to alter the production of acetoin and 2,3-butanediol in *Serratia marcescens* by transforming the species with plasmids containing *vgb.* When the cells were grown on Luria broth supplemented with 2% glucose, the non–*vgb*-bearing strains produced 15-fold as much acetoin and fourfold as much 2,3-butanediol as those cells with *vgb.* For growth on Luria broth supplemented with 2% casein acid hydrolysate, though, a *vgb*-bearing strain produced significantly more of the two products than did

strains without the hemoglobin gene. This result clearly demonstrates the complications that can arise in using bacterial hemoglobins as metabolic engineering tools.

In the area of cell cycle improvements, some of the most complex and important regulatory mechanisms of eukaryotic cells are those that govern cell division. Effective reprogramming of the complex regulatory apparatus to achieve bioprocess goals, such as cessation of proliferation at high cell density to allow an extended period of high production, can require coordinated manipulation of multiple genes. The overexpression of the cyclin-dependent kinase inhibitors p21 and p27 has already proven to be effective in cancer therapy. In a stable genetic configuration, only regulated overexpression of p27 was successful in inducing a sustained Chinese hamster ovary cell growth arrest in G1 phase, which also resulted in a 10-fold increase in per-cell secreted alkaline phospatase, a model heterologous protein used during these studies. Stable overexpression of p21 alone did not result in growth arrest. Recently, by tetracycline-regulated coexpression of p21 and the differentiation factor CCAAT/enhancer-binding protein α (which both stabilizes and induces p21), Fussenegger et al achieved effective cell cycle arrest. Production of secreted alkaline phospatase has been increased 10- to 15-fold, on a per cell basis, relative to an isogenic control cell line. Because the activation of apoptosis response is a possible complication in a proliferation-arrested culture, the survival gene *bcl-*x_L was coexpressed with another CDI, p27, found to enable CHO cell cycle arrest predominantly in G1 phase. CHO cells stably transfected with a tricistronic construct containing the genes for these proteins and for secreted alkaline phospatase showed 30-fold higher secreted alkaline phospatase expression than control cells (25).

BIOMEDICAL APPLICATIONS

Besides manufacturing applications, metabolic engineering is having a significant impact on the medical field. The main focus here is the design of new therapies by identifying specific targets for drug development and by contributing to the design of gene therapies. Such approaches currently target a specific single enzymatic step implicated in a particular disease. There is no assurance, however, that the manipulation of a single reaction will translate to systematic responses in the human body. Although mammalian intermediary metabolism was defined in biochemical terms many years ago, it is important to remember that much of this information accrued from studies in vitro. We have less understanding of the organization in vivo. Furthermore, the intersections of the central pathways of metabolism, such as glycolysis, gluconeogenesis, urea synthesis, tricarboxylic acid cycle, etc, cause changes in one pathway, owing to inborn error or disease, to affect pathways that may seem remote from the initial metabolic defect (73). In this regard, medical applications are no different from the ones mentioned earlier in an industrial context, and, as such, they will benefit from developments

in metabolic engineering through a better analysis of experimental results and applications to the rational selection of targets for medical treatment. In this section, some representative examples are highlighted that illustrate the application of metabolic engineering tools to the study of human disease.

Inborn Errors of Metabolism

One of the earliest applications of MFA to inherited disorders of metabolism concerned aberrations associated with glycogen storage diseases. In a series of ^{13}C-tracer experiments, Kalderon et al (42–45) examined the pathways of hepatic glucose storage and use in glycogen storage disease (GSD) types I (GSD-I) and III (GSD-III). In children with GSD-I, the isotopomer distributions of infused [U-^{13}C]glucose and plasma glucose were identical, indicating absence of glucose recycling, whereas a significant change in the isotopomer distribution of plasma glucose was observed in normal and GSD-III subjects. The absence of recycled glucose in their plasma eliminated a mechanism for glucose production in GSD-I patients involving gluconeogenesis, suggesting a deficiency of a gluconeogenic enzyme such as glucose 6-phosphatase. In contrast, gluconeogenesis was suggested as the major route for endogenous glucose synthesis in GSD-III patients. Moreover, these differences in glucose recycling correlated with significant differences in the glucose C-1 ^{13}C nuclear magnetic resonance splitting pattern in plasma, suggesting that such ^{13}C nuclear magnetic resonance analysis of plasma may be used to noninvasively diagnose defects in gluconeogenesis.

Another type of disorder to which MFA has been applied is hereditary fructose intolerance (HFI), an inborn deficiency in the ability of aldolase B to split fructose-1-phosphate. Continuous exposure of these subjects to parental fructose during infancy may result in liver cirrhosis, mental retardation, and death. In most cases of HFI, final diagnosis of aldolase B deficiency is usually performed in liver biopsy specimens. As an alternative, Gopher et al (27) proposed a method for noninvasive in vivo diagnosis of HFI based on mass isotopomer analysis. In control and HFI children, steps involved in fructose metabolism were quantified by analyzing plasma glucose isotopomer populations after nasogastric infusion of D-[U-^{13}C]fructose. After administration of labeled fructose, the conversion of fructose to glucose was significantly lower in HFI than in control children as determined by this method, supporting its validity as a diagnostic test. Furthermore, it was found that the generally accepted pathway of fructose conversion to glucose, by fructose-1-phosphate aldolase to triose phosphate, accounts for only one-half of the total amount of fructose conversion in normal subjects. It is suggested that a direct pathway from fructose to fructose-1,6-bisphosphate by 1-phosphofructokinase exists, accounting for the remainder of fructose conversion.

Diabetes has also been the subject of many studies using stable isotopomer methods. A series of extensive studies by Cohen (10–12) using streptozotocin-diabetic rats has shown that the increase in relative flux through pyruvate carboxylase and the inhibition of flux through pyruvate kinase that prevents

reconversion of phosphoenolpyruvate into pyruvate may be concerted actions leading to the enhanced gluconeogenesis found in this model of diabetes. Tayek & Katz (90) compared the relative contributions of gluconeogenesis and glycogenolysis with postabsorptive glucose production in normal and non–insulin-dependent diabetes mellitus (NIDDM) humans. They found that total glucose production was elevated in NIDDM patients compared with control subjects and that fractional contribution of gluconeogenesis was comparable, raising doubts about the widely held notion that synthesis of hepatic glycogen is seriously impaired in NIDDM. On the other hand, Landau et al (54) estimated the contribution of gluconeogenesis to glucose production in insulin-dependent diabetes mellitus (IDDM) patients to be significantly less than in normal subjects, also based on analyses of isotopomer distribution. More recently, Peroni et al (70) measured gluconeogenic fluxes in postabsorptive and starved normal and streptozotocin-diabetic rats, in which it was found that the increased gluconeogenic contribution to glucose production in diabetic rats relative to control rats in the postabsorptive state was abolished in the starved state.

Once inherited disorders of metabolism are diagnosed and the affected biochemical pathways have been identified, these diseases may be treated by using genetic-engineering tools. The therapy for many inherited liver enzyme deficiencies requires the removal of toxic intermediate metabolites from the blood of affected individuals. Recent research tries to focus on the removal of circulating toxins by expressing the missing enzymes in tissues other than the liver. This will hopefully positively influence the disease phenotype. Harding et al (29) successfully expressed the phenylalanine hydroxylase (PAH) activity in skeletal and cardiac muscle of mice under the control of the mouse muscle creatine kinase promoter. When they bred the muscle PAH-expressing mice with liver PAH-deficient mice, a progeny was created that lacked PAH activity in liver but expressed PAH in muscle. These mice exhibited hyperphenylalaninemia at baseline, but serum phenylalanine levels decreased significantly when the mice were supplemented with tetrahydrobiopterin, a required cofactor for PAH. This result suggests that gene therapy targeted to heterologous tissue, such as muscle, will be an effective treatment of selected inborn errors of metabolism.

Cell and Organ Physiology

A powerful feature of metabolic engineering is that it allows systematic investigation of metabolic control and regulation in intact tissues. Given that many metabolic disorders, such as liver cirrhosis, post-traumatic hypermetabolism and muscle wasting, cancer cachexia, etc, have no clearly identifiable genetic origins, it is clear that to develop therapeutics or treatment strategies, quantitative characterization has to be achieved at a biochemical level. In this regard, the results of cell or organ physiological studies within the framework of MFA could be very valuable. For example, an important focus of tumor biology has been on understanding the ability of tumors to adapt to adverse growth environments such

as hypoxic or hypoglycemic conditions. It has been suggested that the energy metabolism of tumors is altered in such a way as to accommodate high levels of anaerobic metabolism and alternate substrate utilization. Using AS-30D hepatoma cells, Holleran et al (31) investigated the quantitative importance of acetoacetate and glucose as substrates of energy metabolism in tumors. It was found that acetoacetate diverted pyruvate from pyruvate dehydrogenase (PDH) to pyruvate carboxylation. In contrast, dichloroacetic acid, a metabolic analog of acetoacetate that is an activator of PDH, increased the oxidation of glucose largely through PDH, indicating that PDH is not maximally active in the presence of dichloroacetic acid. Isotopomer spectral analysis of lipid synthesis demonstrated that, in the absence of acetoacetate, glucose supplied 65% of the acetyl-CoA used for de novo lipogenesis. Isotopomer spectral analysis refers to a variant of ^{13}C-isotopomer analysis particularly suited to the study of lipid metabolism (50). In the presence of high levels of acetoacetate, glucose was replaced as a lipogenic precursor, and acetoacetate supplied 85% of the acetyl-CoA for lipogenesis vs only 2% for glucose. Thus AS-30D cells may have a large capacity for acetoacetate use for de novo lipogenesis, leading to greater capacity for fat storage, which may help survival in cachexic conditions.

Portais et al (71) used a mathematical model of the TCA cycle in combination with ^{1}H-^{13}C-nuclear magnetic resonance to calculate the flux distribution in rat brain tumor cells. In their study, it was found that the pyruvate carboxylase activity and the efflux from the TCA cycle in C6 glioma cells are estimated to be very low, suggesting a lack of glutamine production in these cells. In a subsequent publication (72), they reported that glutamine and glucose are metabolized complementarily in C6 cells in that glutamine is mainly used for anaplerosis but not a substrate for energy metabolism, whereas glucose is poorly anaplerotic and is essentially used as energetic fuel. Using a similar method, Bouzier et al (5) found that, unlike normal astrocytes, C6 cells preferentially use lactate as a substrate for oxidative metabolism. Such characterizations of the differences in intermediary metabolism between tumors and their normal counterparts could lead to better understanding of tumor proliferation and may be exploited to control tumor growth in vivo.

MFA has also been used to characterize the effects of acute metabolic stresses, such as hypoxia or reperfusion injury. Malloy et al (62) developed a model based on isotopomer distribution of glutamate in heart tissue extracts to show that, in perfused hearts exposed to a combination of substrates (lactate, acetate, glucose), ischemia-reperfusion leads to an increase in the contribution of acetate and a decrease in the contribution of lactate as sources of acetyl CoA. Ischemia-reperfusion injury also causes an increase in anaplerotic flux. However, exogenously added aspartate or glutamate are not significantly metabolized. This is a finding that establishes a protective role for aspartate and glutamate on myocardial ischemia that does not result from a direct mechanism involving the TCA cycle in the heart tissue. Also in a perfused heart model, Laplante et al (55) established that the cardioprotective effect of fumarate during ischemia or hypoxia occurs through

its reduction to succinate. Another technique developed by Sherry et al (79, 88), showed that lipoamide—an agent being considered to enhance recovery after infarctus—prevented, in large part, the switch from lactate to acetate use induced by ischemia.

Finally, over the past several years, protocols have been developed that allow the extension of ^{13}C-labeling–based flux analysis to noninvasive monitoring of specific organs without biopsies. One such approach involves the use of xenobiotics such as phenylacetate, which in primates is excreted in urine as a glutamine conjugate. Conjugation occurs specifically in the liver. Glutamine is synthesized from α-ketoglutarate via glutamate without rearrangement of carbons. Thus, glutamine carbons reflect the carbons of α-ketoglutarate. Consequently, by analyzing the labeling pattern in the glutamine conjugate in urine, liver metabolic fluxes may be estimated. This method was first validated by Magnusson et al (61), who estimated the fluxes in and around the TCA cycle in human subjects by analyzing the ^{14}C distribution in excreted phenylacetate after infusion with [3-^{14}C]lactate and oral administeration of phenylacetate. Jones et al (40) improved the method by identifying a stable isotope tracer, [U-^{13}C]propionate, which is quantitatively extracted into the liver from portal circulation. Coined as ''chemical biopsy'' by DiDonato et al (17), these noninvasive organ-monitoring protocols used in conjunction with ^{13}C-labeling experiments could become an important research and diagnostic tool, providing a more detailed assessment of the metabolic state of liver in human metabolic disorders.

CONCLUDING REMARKS

The success of biotechnology and biomedical engineering applications of the type addressed in this review—the production of primary and secondary metabolites, the alteration and improvement of cellular properties, and the investigations of the causes and potential treatments of diseases and injuries—are all subject to the understanding of complex networks of metabolic pathways. Because its focus is not on isolated reactions but on the interrelationships of reactions in networks, the emerging field of metabolic engineering should be seen as particularly relevant to these applications. The examples described above clearly demonstrate the breadth of the range of advances realized through metabolic engineering.

Although metabolism and cell physiology provide the main context for analyzing reaction pathways, it should be pointed out that results of flux determination and control have still broader applicability. Thus, besides the analysis of material and energy fluxes through metabolic pathways, the concepts of metabolic engineering are equally useful in the analysis of information fluxes and of those encountered in signal transduction pathways. Because the latter have not yet been well defined, the main focus of this article has been on applications to metabolic pathways. However, once the concepts of information pathways have crystallized, we expect that many of the ideas and tools presented herein will find good use

in the study of the interactions of signal transduction pathways and the elucidation of the complex mechanisms by which external stimuli control gene expression.

Metabolic engineering is the science aiming at a holistic understanding of metabolic functions and cellular physiology. As such it provides a much needed framework for analyzing measurements of differential gene expression obtained through the application of emerging technologies such as DNA microarray hybridization. These data, in combination with detailed flux measurements obtained by available methods and others under development, offer the best promise for a systematic study and elucidation of metabolic networks. As accurate measurements of gene expression, protein content, and in vivo fluxes become readily available, intricate regulatory structures at the genetic and metabolic levels will be gradually better understood using the principles of metabolic engineering. This will have profound implications for the rational modification of metabolic and signaling pathways both in a biotechnological context and in refining current methods for target selection in drug development and gene therapy.

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