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A Chemist's Perspective on the Use of Genetically Engineered Microbes as Reagents for Organic Synthesis

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Introduction

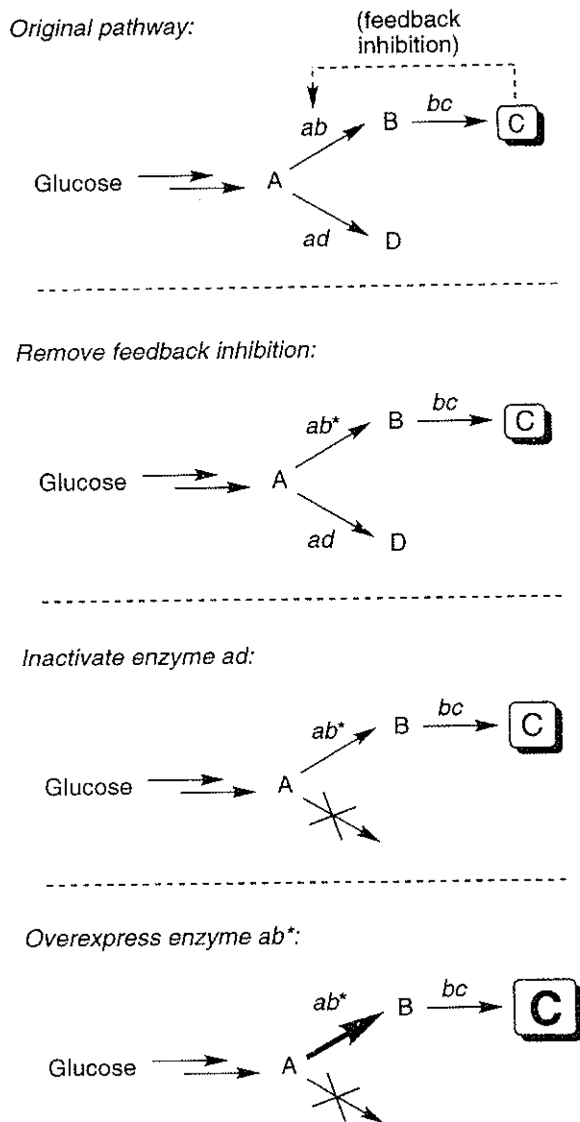
The ever-increasing pressure on chemists to develop processes that are simultaneously enantioselective, high-yielding and environmentally compatible has led to a renewed interest in biocatalysis (Jones, 1986; Crout and Christen, 1989; Davies *et al.*, 1989; Santaniello *et al.*, 1992; Ikemi, 1994). From a chemist's perspective, enzymes possess a number of properties that make them attractive alternatives to traditional reagents, many of which are toxic and require the use of organic solvents that pose ever-increasing disposal problems. By contrast, enzymes normally operate in aqueous solution and are completely biodegradable. Moreover, they combine efficiency with regio- and enantioselectivity at levels that are rarely achieved by non-biological catalysts. Finally, nature has provided a very large 'library' of enzymes dispersed among living organisms, and it is usually possible to find an enzyme that catalyzes a desired chemical reaction by screening sufficiently large numbers of organisms, especially bacteria and fungi.

There are two general situations in which enzymes can be advantageously applied to organic synthesis. If the desired compound can be produced by a naturally-occurring metabolic pathway or one that can be created by adding one or more foreign enzymes to an existing metabolic pathway, whole cells can be used to convert inexpensive carbon and nitrogen sources into the end product without the need for 'chemical' steps. This strategy has been successfully applied to the synthesis of amino acids, vitamins and even some high-volume industrial chemicals. On the other hand, if the desired compound does not occur in nature, enzymatic catalysis can be used to augment traditional organic synthesis, allowing one to synthesize the starting material in an environmentally compatible manner or to replace a particularly difficult chemical step.

Unfortunately, it is not always straightforward to apply enzymatic catalysts to

Camille and Henry Dreyfus New Faculty Awardee, 1994 – 1999

Biotechnology and Genetic Engineering Reviews – Vol. 14, April 1997
0264-8725/97/14/67-143 $20.00 + $0.00 © Intercept Ltd. P.O. Box 716, Andover, Hampshire SP10 1YG, UK



Scheme 1

become clear, metabolic engineering is an iterative process, with each alteration incrementally increasing the yield of the desired product. Unfortunately, even with detailed information on rate constants and levels of enzymes and their substrates, it is very difficult to predict which alterations will have the largest effects on productivity. One therefore relies on experience and judgment to determine the order in which alterations in a biosynthetic pathway will be made. The sequence of steps usually mirrors that presented below, although the specifics may vary.

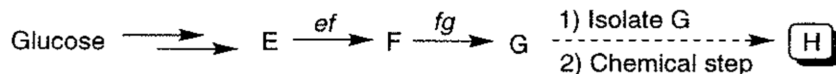
To remove feedback inhibition by the end product, a gene encoding a mutant version of enzyme *ab* (*ab**) is introduced into the cell, where it replaces the wild-type copy. These mutations are typically single amino acid changes that alter the binding affinity of the enzyme for the inhibitor. Such mutations are often derived from classical strain improvement studies. To further increase the carbon flux toward the desired product, the enzyme that converts A into D (enzyme *ad*) is removed by inactivating its corresponding gene. Finally, the level of the enzyme catalyzing a potentially rate-limiting step in the biosynthesis (enzyme *ab**) can be increased by increasing the gene dosage in the organism or by using a stronger promoter. Clearly, other steps could be targeted as well, and one generally stops the engineering when the strain's productivity makes the overall process economically feasible.

While this simple example illustrates the general strategies of metabolic engineering, real systems are more complex in several important ways. Most importantly, real metabolic pathways are rarely limited by single enzymes (the so-called 'bottleneck' situation); instead, all of the enzymes along a pathway are more or less equally rate-limiting (Krämer, 1996; Niederberger *et al.*, 1992). The bottleneck situation usually holds true only for enzymes that at the beginning of a pathway that are subject to feedback inhibition. Once this inhibition is disabled by mutations, flux control through the pathway is distributed throughout the steps. Thus, it is often found that significantly overexpressing a single enzyme in a pathway improves productivity by only a relatively small amount (usually around two to five-fold, (Bailey, 1991)). In addition, our simple example neglects transport of the metabolite out of the cell. This is a complex process in which both passive and active transport must be considered. Finally, in addition to focusing on the enzymes involved in the specific metabolic pathway of interest, one must also consider the possibility that the supply of key precursors from central metabolism might also be limiting. This last point is extremely important in the production of aromatic amino acids by fermentation (*vide infra*). All of these problems could in principle be identified by determining the concentrations of the various metabolites in a living cell under various growth conditions. Recent advances in using isotopically-labeled media in conjunction with high-resolution NMR are opening many possibilities in this area (Krämer, 1996; Sonntag *et al.*, 1995).

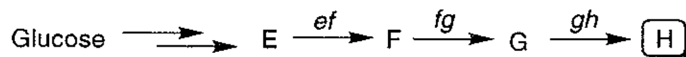
EXPANDING AND REDESIGNING METABOLIC PATHWAYS

With the general principles of metabolic engineering in mind, the design of new metabolic pathways is relatively straightforward. To illustrate this, consider the metabolic pathways found in two different microbes (*Scheme 2*). This example is a simplified version of antibiotic production by semi-synthesis. The final product, H, can be derived from G by a chemical step after its production by Microbe #1. However, another organism (Microbe #2) has an extended pathway that directly yields H in a single fermentation. Why not simply use Microbe #2 for the fermentation? There are a number of motivations for keeping the original strain, including the high productivity that has been achieved for G by strain improvement, the many years of fermentation experience with this organism and issues of regulatory compliance. For this reason, it would be more desirable to extend the biosynthetic pathway of Microbe #1 by including enzyme *gh* from Microbe #2. This can be accomplished by expressing this enzyme in Microbe #1.

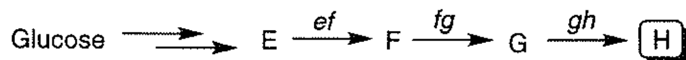
Original pathway, Microbe #1:



Enzyme from Microbe #2:



Express enzyme gh in Microbe #1:



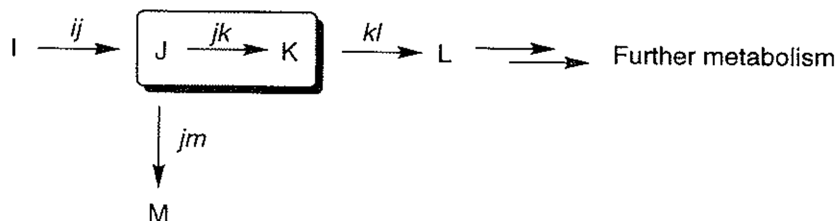
Scheme 2

The ability to combine enzymes from various organisms and create hybrid pathways that do not exist in nature is one of the most powerful aspects of metabolic engineering. By coupling these processes with simple chemical steps, the synthesis of a wide range of commercially-important products becomes possible. Several examples of this approach have been published, although none of the genetically engineered organisms have been used on an industrial scale. The major difficulties in this approach are similar to those outlined above: multiple enzymes conspiring to limit the carbon flux through metabolic pathways, limitations in precursor supply, etc.

ENGINEERED MICROBIAL CELLS AS SUBSTITUTES FOR ISOLATED ENZYMES

While the two approaches described above are very powerful, they are not applicable to the synthesis of compounds that are not natural metabolites or simple derivatives. Completely biological routes to many industrially-important compounds are difficult to envision. For this reason, routes based on traditional organic synthesis will continue to play important roles in industrial chemical manufacturing. However, the increasing demand for cleaner chemistry and the focus on synthesizing pharmaceuticals and agrochemicals as pure enantiomers provides natural roles for enzymatic steps within the framework of organic chemistry. Consider the hypothetical metabolic pathway in *Scheme 3*. This arrangement is typical of assimilative metabolic pathways in bacteria. In this example, the natural role of enzyme *jk* is to catalyze the interconversion of J and K so that I can be utilized as a carbon source for growth of the organism. Of more interest to a chemist, the same enzyme also catalyzes similar reactions on a wide variety of substrates with high enantioselectivities. For this reason, enzyme *jk* would be useful as a general synthetic reagent. Without metabolic engineering, applications of enzyme *jk* to synthesis would require the use of the purified enzyme or whole cells of the organisms that express the complete metabolic pathway.

Original pathway:



Express enzyme jk in easily-handled host:

Scheme 3

The use of whole microbial cells as chemical catalysts is experimentally simple (Faber, 1995), but it can be frustrated by side-reactions and overmetabolism. In the example shown in *Scheme 3*, J can be metabolized by both enzymes *jk* and *jm*. While conditions can sometimes be arranged to favor the desired enzyme (by structural variations of the substrate, random mutagenesis to inactivate competing enzymes, etc.), the presence of multiple enzymes that accept the same substrate remains a difficult problem. Over-metabolism of the desired product can also occur, particularly when the compound of interest structurally resembles a natural metabolite. This is shown by the conversion of K to L, which in turn enters primary metabolism.

To avoid these problems, an enzyme can be isolated and used in pure form to catalyze synthetic reactions. Using purified enzymes obviates problems with competing enzymes and overmetabolism, but these benefits must be carefully weighed against several disadvantages that include (1) the time and expense required for protein isolation; (2) limited stability with respect to pH and temperature; (3) reliance on expensive cofactors; (4) localization of some useful enzymes in phospholipid membranes. While several ingenious approaches have been used to alleviate these problems, including immobilization (Katchalski-Katzir, 1993), cross-linked enzyme crystals (St Clair and Navia, 1992; Lalonde *et al.*, 1995) and the use of organic solvents in place of water (Klibanov, 1990; Koskinen and Klibanov, 1996) only a few purified enzymes – most notably lipases and proteases – have achieved wide acceptance as synthetic reagents.

To combine the advantages of using isolated enzymes as synthetic reagents with the experimental simplicity of whole-cell mediated reactions, we (Stewart *at al.*, 1996a,b) and others (Zylstra amd Gibson, 1991; Herrmann *et al.*, 1994a,b) have engineered easy-to-handle microorganisms so that they express synthetically-useful enzymes. In the example shown in *Scheme 3*, the gene for enzyme *jk* would be inserted into a new microbial host that lacks the original metabolic pathway. In effect, the engineered cells act as the synthetic equivalent of the isolated enzyme.

The cells provide a constant source of enzyme as well as any required cofactors. While a good deal of biochemical and microbiology experience is involved in *creating* these catalysts, this experience is not necessary in order to *apply* them to solving synthetic problems. This strategy is explicitly designed to make enzymatic catalysis accessible to the practicing chemist.

CHOICE OF HOST CELLS

Plants, bacteria and fungi have all been used for metabolic engineering studies. In the case of plants, most the work is aimed at altering the composition of oils and triglycerides found in their seeds (Knauf, 1987; Ohlrogge, 1994; Moffatt, 1995; Töpfer *et al.*, 1995). The goals in this area range from creating margarine with lower saturated fatty acid content to the creation of temperate plants that produce fatty acids that are currently derived from tropical sources. While this is an important area of research, metabolic engineering of bacteria and fungi is likely to have a greater impact on organic chemistry in the short term, mostly due to the great deal of industrial experience in fermentation and the fact that microbial cells can be used in the laboratory with existing apparatus and techniques.

Two bacteria currently dominate metabolic engineering: *E. coli* and *Corynebacterium glutamicum* (as well as its close relatives *C. flavum* and *C. lactofermentum*). Interestingly, *E. coli* was not considered an industrial organism until recently. However, the ease of gene cloning in this bacterium, its lack of pathogenicity and the large amount of accumulated metabolic and genetic data make up for the lack of industrial experience in using this organism. On the other hand, the use of *C. glutamicum* for industrial-scale amino acid production goes back nearly 40 years (Kinoshita *et al.*, 1957). In addition, the metabolic pathways of *C. glutamicum* are generally simpler and less redundant than those in *E. coli*, and metabolite overproduction is well-established in this host (Jetten and Sinskey, 1995). However, these advantages must be balanced against the much more rudimentary cloning technology that is available for this organism (Sano, 1994) and the relative paucity of quantitative information on metabolism in the unmodified organism (Krämer, 1996). While *E. coli* and *C. glutamicum* are the major organisms for metabolic engineering, *Streptomyces*, *Serratia marcescens* and other species have been used to solve specific problems.

Baker's yeast (*Saccharomyces cerevisiae*) has been the most commonly-used fungal host for genetically engineered biocatalysis. The molecular genetics of this organism are also well-studied and the cloning methodology is also highly-developed. In addition, this organism has been extensively used in organic chemistry as a chiral reducing agent for ketones, keto-esters and keto-acids (Servi, 1990; Csuk and Glanzer, 1991). It should be noted that modern-day baker's and brewer's yeasts are unusual organisms, the results of many years of selective pressure for rapid growth and high production of carbon dioxide and/or ethanol. For this reason, glycolysis occupies a very prominent place in yeast metabolism, and some common strains of yeast devote 30% of their total cellular protein to glycolytic enzymes. The eukaryotic nature of yeast can be advantageous for metabolic engineering since these organisms have many of the same cellular compartments and organelles as higher plants and animals. While most metabolic engineering of fungi has focused on baker's yeast, other fungi are more appropriate for specific projects. For example, in re-engineering the biosyn-

thesis of β-lactams that are used as the starting materials for the semi-synthesis of antibiotics, fungi that produce the basic penicillin nucleus are the preferred hosts.

SYNTHESIS OF NATURAL PRODUCTS BY RECOMBINANT MICROBES

In terms of economic impact, the use of recombinant microbes for synthesizing natural products, especially amino acids, is by far the most important application of this technology. Annual production of L-glutamate is estimated at 500,000 tons; that of L-lysine is estimated at approximately 200,000 tons (Krämer, 1996). Other amino acids produced by industrial-scale fermentations include L-phenylalanine, L-tryptophan, L-threonine and L-isoleucine. Glutamate (in the form of its monosodium salt) is primarily used as a flavor enhancer in food and phenylalanine is used in the manufacture of Aspartame®, the artificial sweetener. Tryptophan, lysine, threonine and isoleucine are used to supplement animal nutrition since typical feeds (defatted seeds and grains) are especially low in these essential amino acids. (While animal feeds are also low in methionine, this amino acid is produced by chemical synthesis and added in recemic form. The tight integration of methionine into one-carbon metabolism will make it very difficult to overproduce this amino acid without its rapid degradation by other cellular pathways.) However, in addition to the intrinsic commercial interest, the results of these strain engineering studies have contributed much to our understanding of how metabolism is integrated, especially under conditions of high stress. This metabolic knowledge has been applied in designing microbial routes to other products, both natural and unnatural.

AMINO ACIDS

Production of amino acids by bacterial fermentation began in 1957 with the discovery that cells of *Micrococcus glutamicum* secreted large amounts of L-glutamate in response to various environmental stresses (Kinoshita *et al.,* 1957). Later, strain engineering was applied to this organism, renamed *Corynebacterium glutamicum*, to produce L-lysine (Kinoshita *et al.*, 1958). Traditional strain improvement techniques were used to create various *Corynebacter* strains that overproduced specific amino acids and many of these were used commercially. In general, this involved treating the microbes with non-specific mutagens, then growing the cells in the presence of a non-metabolizable amino acid analog (*e.g.*, 4-fluorophenylalanine or *S*-(2-aminoethyl)-cysteine, which are phenylalanine and lysine analogs, respectively). These analogs are toxic to wild-type cells because they bind to the feedback regulation sites on biosynthetic enzymes and inhibit catalysis, although the analogs are not accepted for protein synthesis. Resistance to these analogs usually occurs by disabling feedback inhibition on biosynthetic enzymes, which is obviously advantageous in overproducing amino acids. With the advent of molecular cloning technology, there was a renewed interest in creating even better production strains by rational re-engineering of metabolic pathways. This has involved combining feedback-resistant genes that were initially found in different strains into a single cell, as well as the overproduction of specific biosynthetic enzymes that were identified as bottlenecks in the process. Because the primary focus of this review is to show how these strains can augment traditional organic chemistry, neither the strategies involved in strain engineering nor the details

of their creation will be covered in detail here. Instead, a broad overview of the work in this field will be presented so that a clear picture of its successes and shortcomings relative to traditional organic processes can be discerned. In addition, only the highest level of production achieved by a research group will be reported; in most cases, this was the culmination of a number of iterative steps that are detailed in the original papers. It should be borne in mind that many different sets of cultivation and production conditions were used to collect these data and direct comparisons of product yields should therefore be made with caution.

Microbial metabolism and amino acid overproduction

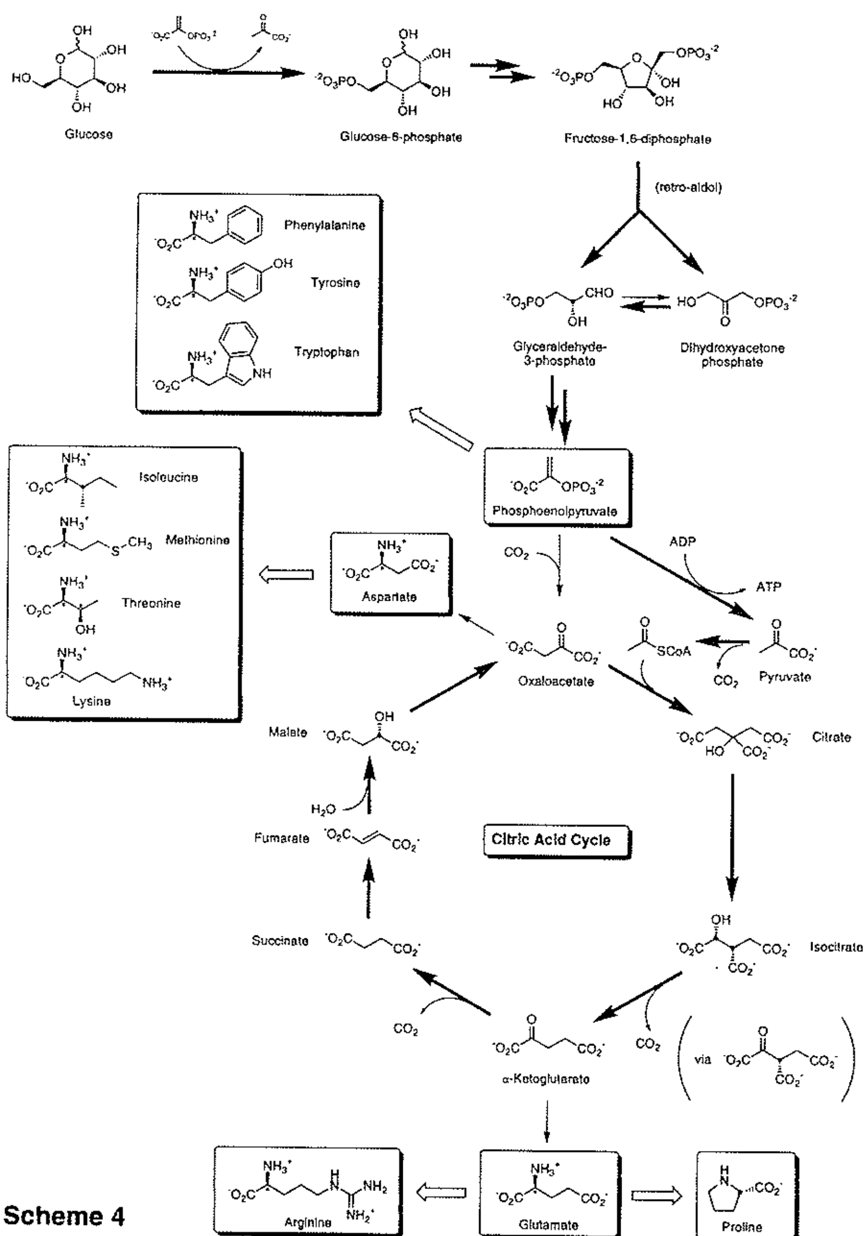
While a detailed description of metabolism is beyond the scope this article, a general understanding of carbon flow in central metabolism is a prerequisite to understanding how it can be advantageously manipulated. *Scheme 4* shows a simplified version of central metabolism in a bacterium such as *E. coli* or *C. glutamicum*. This representation emphasizes the carbon flow and, for the sake of clarity, does not show the participation of cofactors in many steps. This practice will be followed throughout this paper. *Scheme 4* also shows how the various amino acids are derived from key metabolic intermediates and their competition for carbon flow at each branch. The major pathway for carbon flux is indicated by heavy lines.

Under normal conditions, only small amounts of central intermediates are converted into amino acids and their biosynthesis is closely matched to cellular needs. However, when bacterial metabolism is highly unbalanced, either by exogenous stresses or by deliberate modifications in the levels of biosynthetic enzymes, a phenomenon known as 'uncoupling' or 'energy spilling' is observed. When carbon sources are in large excess, but the bacteria are growing slowly or not at all, the cells rid themselves of excess energy by dramatically enhancing amino acid production or by activating futile reaction cycles in other pathways (Krämer, 1996). These unbalanced conditions are precisely those sought for large-scale amino acid production. However, this phenomenon must be kept in mind, since the state of cellular metabolism during amino acid hyperproduction may be quite different from that under normal conditions. For this reason, unanticipated limitations on production may be observed, such as rate-limiting synthesis or recycling of precursors and cofactors. This again underscores the importance of determining metabolic states of cells under production conditions directly, rather than extrapolating from measurements made under physiological conditions.

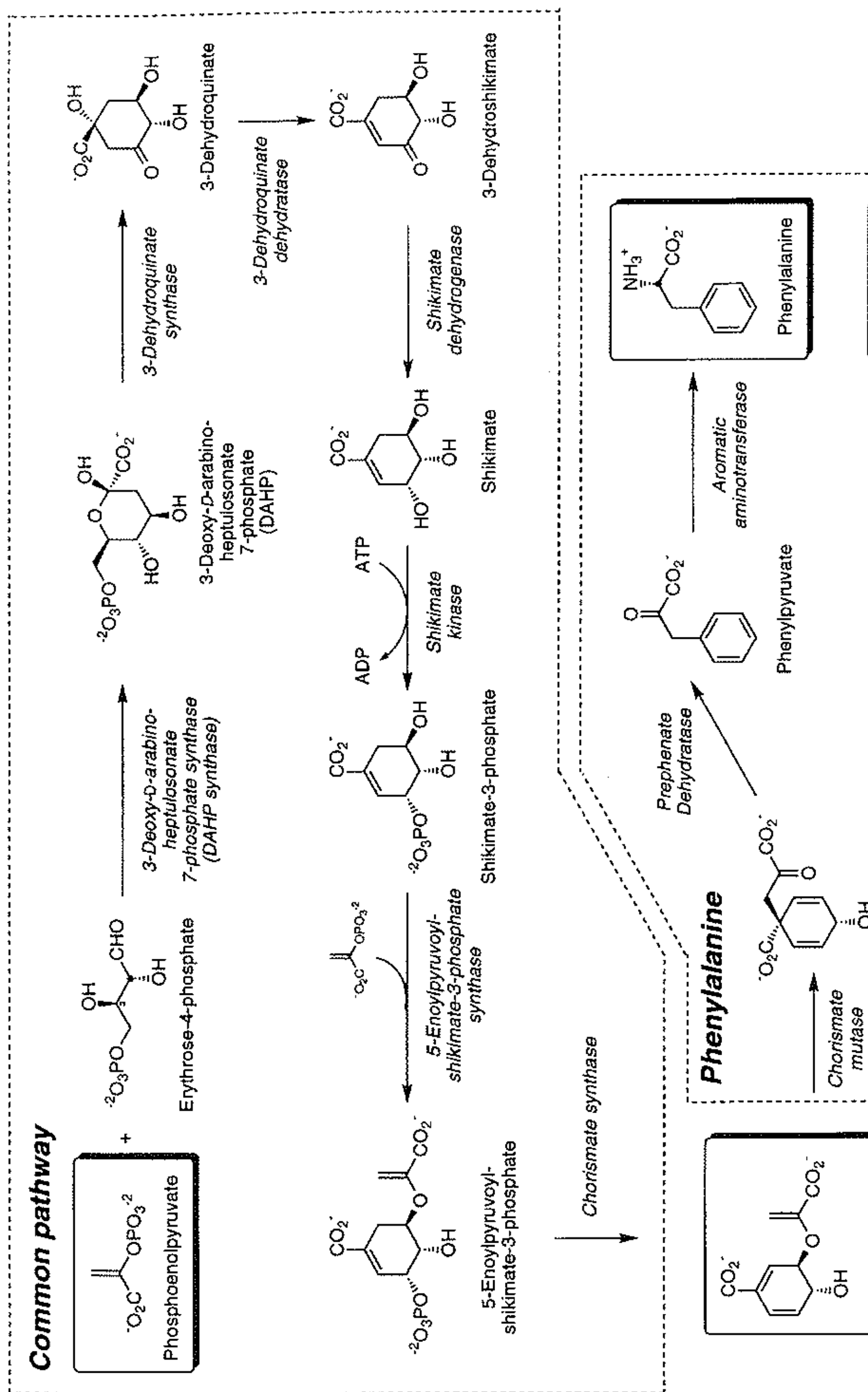
From *Scheme 4*, it is apparent that the amino acids targeted for overproduction can be logically divided into three categories: the aromatic amino acids (phenylalanine and tryptophan), the aspartate family (lysine, threonine and isoleucine) and the glutamate family (proline and arginine). This order of presentation has been designed to show how carbon flows interconnect between the major pathways and to illustrate why, for example, a strain that overproduces lysine would be the best starting point for constructing an isoleucine producer.

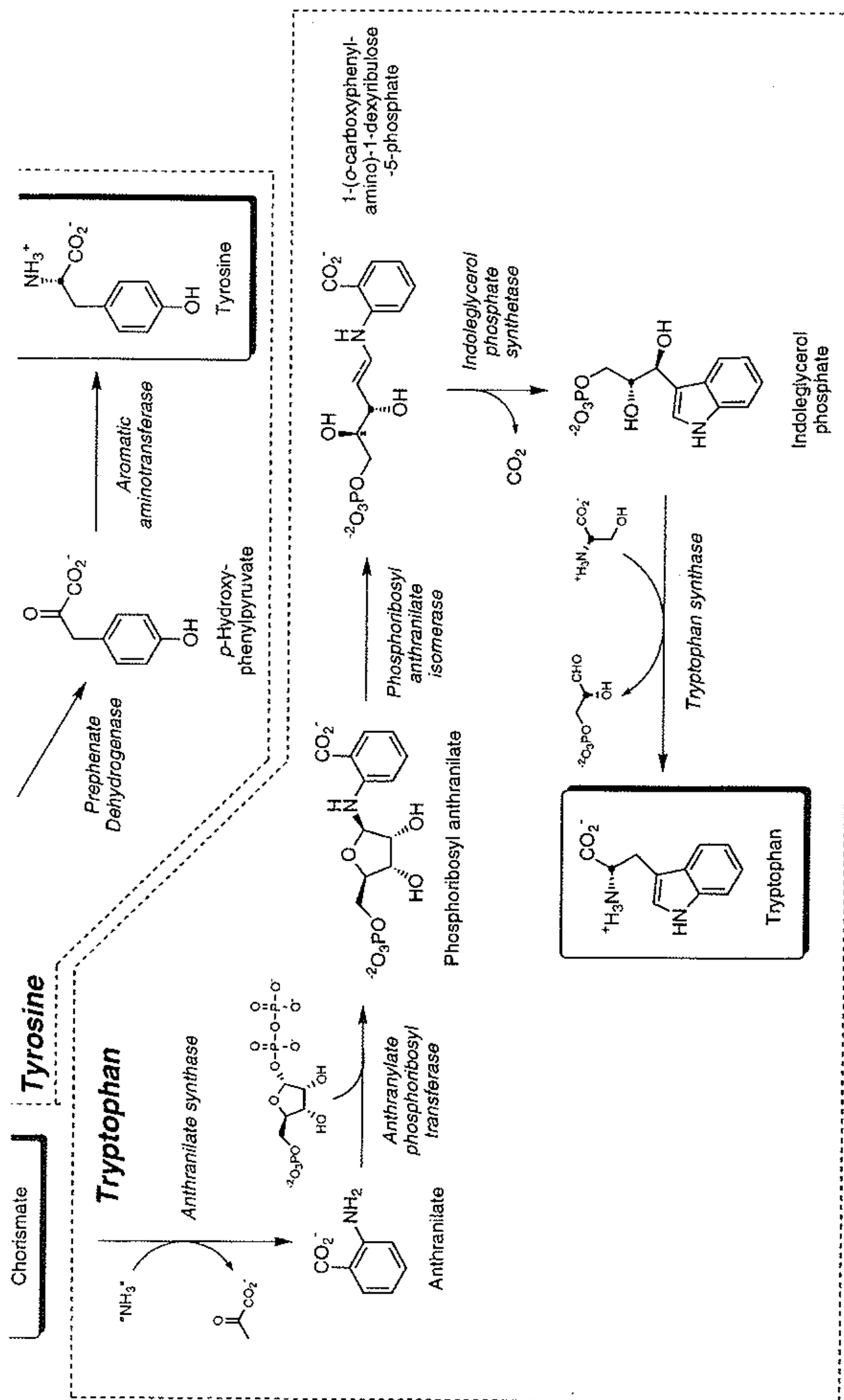
Engineering the common pathway of aromatic amino acid biosynthesis

Aromatic amino acids are synthesized by bacteria by a multi-step route from two



Scheme 4


Common pathway
Phosphoenolpyruvate
Erythrose-4-phosphate
3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHP synthase)
3-Deoxy-D-arabino-heptulosonate 7-phosphate (DAHP)
3-Dehydroquinate synthase
3-Dehydroquinate
3-Dehydroquinate dehydratase
3-Dehydroshikimate
Shikimate dehydrogenase
Shikimate
ATP
ADP
Shikimate kinase
Shikimate-3-phosphate
5-Enoylpyruvoyl-shikimate-3-phosphate synthase
5-Enoylpyruvoyl-shikimate-3-phosphate
Chorismate synthase
Phenylalanine
Chorismate mutase
Prephenate Dehydratase
Phenylpyruvate
Aromatic aminotransferase
Phenylalanine



Scheme 5

central metabolites, phosphoenolpyruvate and erythrose-4-phosphate (Pittard, 1987). In order to understand how each of the amino acids can be individually overproduced, one must consider the common pathway from which they are derived (*Scheme 5*). The committed step in aromatic amino acid biosynthesis is catalyzed by deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthase, which catalyzes the aldol condensation between phosphoenolpyruvate and erythrose-4-phosphate. After several steps, another molecule of phosphoenolpyruvate is added to shikimate-3-phosphate to afford 5-enolpyruvoyl-shikimate-3-phosphate. Chorismate is formed by the *cis*-elimination of phosphate and represents the end of the common pathway.

There are several aspects of this pathway that are important for understanding amino acid overproduction. The expression of all the enzymes is coordinately regulated in wild-type bacteria. High concentrations of aromatic amino acids decrease gene expression. In addition, the kinetic properties of DAHP synthase are also regulated by feedback inhibition by aromatic amino acids. This enzyme is a major control point in regulating carbon flux through the common pathway. *E. coli* actually contains three different forms of DAHP synthase, each of which is subject to feedback inhibition by one of the three aromatic amino acids. The situation is slightly different in *C. glutamicum*. This organism has only a single DAHP synthase that is subject to a complex pattern of feedback inhibition by the end products. In addition, *C. glutamicum* DAHP synthase is part of a bifunctional enzyme that catalyzes both the synthesis of DAHP as well as the Claisen rearrangement of chorismate, the first committed step in the biosynthesis of phenylalanine. A key aspect of aromatic amino acid biosynthesis is its heavy dependence on phosphoenolpyruvate. Note that two molecules of phosphoenolpyruvate are consumed for every molecule of chorismate synthesized. For this reason, flux through the common pathway of aromatic amino acid biosynthesis depends greatly on the supply of this metabolite.

The common pathway of aromatic amino acid biosynthesis has been extensively studied with the aim of increasing production of aromatic amino acids and other products that can be derived by diverting intermediates into designed metabolic pathways. Because of its role as a gatekeeper for the pathway, a feedback-resistant version of DAHP synthase is overexpressed in all strains that overproduce aromatic amino acids. However, analysis of carbon flux showed that supplies of phosphoenolpyruvate and erythrose-4-phosphate limit the carbon flux when a feedback-resistant version of DAHP synthase was expressed in *E. coli* at a high level (Patnaik and Liao, 1994; Patnaik *et al.*, 1995). While many cellular enzymes compete with DAHP synthase for the available phosphoenolpyruvate, the phosphotransferase system that transports glucose with concomitant phosphorylation at the C-6 hydroxyl (*Scheme 4*, first step) is the most important. According to the stoichiometry of *Scheme 4*, each glucose molecule gives rise to two molecules of phosphoenolpyruvate; however, the import of the sugar consumes one of these. Thus, high glucose transport starves DAHP synthase for one of its substrates. Several methods have been devised to overcome this problem in *E. coli*, including the development of a glucose import pathway that utilizes ATP as the phosphate donor rather than phosphoenolpyruvate (Flores *et al.*, 1996), the use of xylose as a carbon source rather than glucose (since xylose import is accompanied by phosphorylation by ATP (Patnaik *et al.*, 1995)) and the overexpression of phosphoenolpyruvate synthase (Patnaik and Liao, 1994). The latter enzyme catalyzes the synthesis of phosphoenolpyruvate at the expense of ATP: pyruvate +

ATP → phosphoenolpyruvate + AMP + P_i. In addition to limiting phosphoenolpyruvate, Frost and co-workers have shown that the cellular supply of erythrose-4-phosphate can also limit flux through the common pathway under certain conditions (Draths *et al.*, 1992a). This was relieved by overexpressing transketolase, which catalyzes the key step in the synthesis of this metabolite from glucose *via* the pentose phosphate pathway (Draths *et al.*, 1992a). These workers have also shown that the level of 3-dehydroquinate synthase (*Scheme 5*, second step) must also be increased under high carbon flux conditions to avoid a bottleneck situation (Dell and Frost, 1993). Recently, a number of alterations that increased carbon flow through the common pathway of aromatic biosynthesis were stably combined into a single *E. coli* strain (Snell *et al.*, 1996).

Phenylalanine

Phenylalanine is synthesized from chorismate in three steps (*Scheme 5*). The rearrangement of chorismate to prephenate is the committed step in the biosynthesis of both phenylalanine and tyrosine. As one of two examples of an enzyme-catalyzed pericyclic reaction, this apparent Claisen rearrangement has attracted a great deal of attention from chemists and biologists that recently culminated in the publication of crystal structures for this enzyme from three different sources (Chook *et al.*, 1993; Xue *et al.*, 1994; Lee *et al.*, 1995a,b). Once formed, prephenate is aromatized by dehydration to phenylpyruvate in the final committed step to phenylalanine. There have been four reports in which molecular cloning techniques were used to significantly overproduce phenylalanine, and various *Corynebacter* strains were used as the hosts for all of these studies (*Table 1*) (Ito *et al.*, 1990; Ozaki *et al.*, 1985; Ikeda *et al.*, 1992; Ikeda and Katsumata, 1993). Similar strategies were used by all four groups to increase phenylalanine production: overexpression of a feedback-resistant chorismate mutase that was originally created by traditional strain improvement techniques. The *Corynebacter* hosts had previously been subjected to strain improvement to increase carbon flux into the aromatic amino acid biosynthetic pathway. To increase further the commitment to this pathway, Ito and co-workers (1990) and Ikeda and Katsumata (1993) also co-expressed a feedback-resistant DAHP synthase. These show how rational engineering can be advantageously combined with traditional strain improvement to solve problems in chemical production.

Tryptophan

Tryptophan is synthesized from chorismate by a multi-step pathway that commences with the conversion of chorismate to anthranilate by anthranilate synthase (*Scheme 5*, bottom). This enzyme is subject to end-product inhibition and thereby controls carbon flow into this pathway. Once formed, anthranilate is coupled with phosphoribosyl pyrophosphate, which then undergoes an enzyme-catalyzed isomerization to an enamine. Following indole ring formation, tryptophan synthase catalyzes formation of tryptophan from indole (formed by a retro-aldol fragmentation of indoleglycerol phosphate) and serine. Free indole is not released during this reaction, but instead remains enzyme-bound.

Because of its economic importance, there have been a number of approaches to

Table 1. Production of phenylalanine by engineered *Cornynebacter* strains

Strategy	Yield (g/l)	Reference
Overexpressed feedback-resistant *C. lactofermentum* prephenate dehydratase and DAHP synthase in *C. lactofermentum*	18	Ito *et al.*, (1990)
Overexpressed feedback-resistant *C. glutamicum* chorismate mutase in *C. glutamicum*	19	Ozaki *et al.*, (1985)
Overexpressed feedback-resistant *E. coli* chorismate mutase/prephenate dehydratase in *C. glutamicum*	23	Ikeda *et al.*, (1993)
Overexpressed feedback-resistant *C. glutamicum* DAHP synthase, chorismate mutase and prephenate dehydratase in *C. glutamicum*	28	Ikeda and Katsumata (1992)

creating fermentation routes to tryptophan (*Table 2*) (Tribe and Pittard, 1979; Aiba *et al.*, 1980, 1982; Azuma *et al.*, 1993; Matsui *et al.*, 1988; Katsumata and Ikeda, 1993). Bacteria regulate tryptophan production at several levels, including feedback inhibition at the entry to the common pathway (DAHP synthase) and the committed step to tryptophan biosynthesis (anthranilate synthase) as well as coordinate regulation of the levels of the tryptophan pathway enzymes. Successful overproduction by rational metabolic engineering evolved over time and the impediments were iteratively addressed (Krämer, 1996). The strategies are summarized in *Table 2*. It should be noted that high levels of overproduction by engineered *E. coli* strains required that anthranilate be added to the culture during amino acid production; this requirement detracts from the attractiveness of these strains from an economic standpoint. On the other hand, the *C. glutamicum* strain reported by Katsumata and Ikeda (1993) produces a high level of tryptophan directly from sucrose and ammonia and does not require the addition of any biosynthetic intermediates to the fermentation.

The approach of Yokota and co-workers (1992) deserves special comment. In this case, an *E. coli* strain was constructed that overexpressed *Enterobacter aerogenes* tryptophanase, an enzyme that catalyzes tryptophan degradation into indole and serine under normal cellular conditions. To use this strain for tryptophan production, cells were grown, then transferred to a buffer containing indole, pyruvate and ammonia. (Pyruvate and ammonia are converted to serine by *E. coli* serine dehydratase.) In this case, the cells were used as the source of two enzymes – tryptophanase and serine dehydratase – and the rest of their metabolism was irrelevant.

Lysine

The biosynthetic pathways leading to lysine, methionine, threonine and the branched amino acids commence with the side-chain phosphorylation of aspartate catalyzed by aspartyl kinase (*Scheme 6*, middle) (Cohen and Saint-Girons, 1987). This step is the major point of regulation in lysine and threonine biosynthesis. *E. coli* contains three separate aspartyl kinases, each subject to feedback inhibition by the end products (lysine, methionine and isoleucine). In *C. glutamicum*, there is only a single aspartyl kinase that is cooperatively inhibited by threonine and lysine. Once formed, the mixed anhydride is then reduced to aspartate semialdehyde, which then undergoes an aldol reaction with pyruvate that is followed by cyclization to dihydropicolinate. This is the first committed step in lysine biosynthesis. In *E. coli*, this enzyme is also subject to end-product inhibition; however, this is not the case in *C. glutamicum*. After reduction of the carbon-carbon double bond, the liberated α-amino group of tetrahydropicolinate is acylated. Although succinate is shown as the acyl group, some bacteria utilize acetate in its place. The second nitrogen is then added and the acyl group is removed to afford L,L-diaminopimelate. This is epimerized to the *meso* isomer, then decarboxylated to give lysine. *C. glutamicum* also has a second pathway leading to *meso*-diaminopimelate that is absent in *E. coli*: *meso*-diaminopimelate dehydrogenase catalyzes the direct conversion of tetrahydropicolinate to *meso*-diaminopimelate (Ishino *et al.*, 1987, 1988).

Virtually all the lysine sold commercially is produced by *C. glutamicum* strains that were created by traditional strain improvement techniques. Historically, lysine was the second amino acid overproduced by a *C. glutamicum* strain (Kinoshita *et al.*,

Table 2. Production of tryptophan by engineered microbial strains

Strategy	Host	Yield (g/l)	Reference
Combined feedback-insensitive *E. coli* DAHP synthase and anthranilate synthase mutants with elevated levels of tryptophan pathway enzymes	*E. coli*	1.3	Tribe and Pittard (1979)
Overexpressed *E. coli* tryptophan pathway enzymes in which anthranilate synthase and anthranilate phorphoribosyltransferase transferase enzymes were feedback-insensitive. Also disabled tryptophan repressor and tryptophanase from the host cells. Added anthranilate to the culture medium during tryptophan production phase	*E. coli*	6.2	Aiba *et al.*, (1980, 1982)
Used the same system as in the previous entry; however, the tryptophan pathway enzymes were randomly mutagenized to further increase their resistance to feedback inhibition. Added anthranilate to the culture medium during tryptophan production phase and used a detergent to induce tryptophan crystallization during fermentation	*E. coli*	55	Azuma *et al.*, (1993)
Overexpressed *Enterobacter aerogenes* tryptophanase in *E. coli* and used the grown cells to catalyze tryptophan synthesis by adding indole, pyruvate and ammonia	*E. coli*	18	Kawasaki *et al.*, (1995)
Overexpressed *Corynebacterium lactofermentum* tryptophan pathway enzymes in which both anthranilate synthase and tryptophan synthase were feedback-resistant in a strain that already overproduced tryptophan and phenylalanine	*C. lactofermentum*	5.7	Matsui *et al.*, (1988)
Overexpressed feedback-resistant DAHP synthase as well as the tryptophan pathway enzymes in which anthranilate synthase and anthranilate phorphoribosyltransferase had been randomly mutated to remove feedback inhibition	*C. glutamicum*	43	Katsumata and Ikeda (1993)

1958). No rationally engineered *E. coli* or *C. glutamicum* variant has come close to the levels of lysine secretion achieved by the existing production strains. However, the disadvantage of strains created by random mutagenesis is that they contribute little or no guidance for redesigning other metabolic pathways.

Since feedback inhibition of aspartyl kinase by lysine and threonine is the major point of regulation, mutations that weaken this interaction would be expected to increase lysine production. These can be created by random mutagenesis of *C. glutamicum*, followed by growth in the presence of *S*-(2-aminoethyl)-cysteine and threonine (Schrumpf *et al.*, 1992). Recently, it has also been shown that the efficiency of lysine transport is also increased in overproducing strains and that this is a key property that must be considered in strain engineering (Schrumpf *et al.*, 1992; Kele *et al.*, 1996). Attempts to further increase lysine production in a *Corynebacterium lactofermentum* strain bred by a traditional strain improvement approach by overexpressing a feedback-resistant aspartyl kinase gave no significant improvement in lysine production (27.3 vs. 25.9 g/l) (Jetten *et al.*, 1995). However, co-expression of *C. glutamicum* dihydropicolinate synthase and a feedback-resistant aspartyl kinase in a *C. glutamicum* host bred for lysine production resulted in a further 1.5-fold increase in lysine production to a level of 10 g/l (Cremer *et al.*, 1991). To address the possibility that an insufficient supply of aspartate might be limiting in lysine overproduction, fumarate was added during fermentation with a *C. glutamicum* strain that expressed a feedback-resistant aspartyl kinase (Menkel *et al.*, 1989). This citric acid cycle intermediate was expected to increase levels of aspartate (*Scheme 4*), which in turn would direct more carbon flow to lysine. While a modest increase in lysine production was observed, the strain also secreted malate and succinate, suggesting that the enzymes required to convert fumarate into aspartate were unable to cope with the high level of substrate (*Scheme 4*). To alleviate this problem, *E. coli* aspartase was expressed in this *C. glutamicum* strain since this enzyme interconverts fumarate plus ammonia and aspartate. The strain expressing aspartase and a feedback-resistant aspartyl kinase produced 10 g/l lysine when fumarate was added to the growth medium (Menkel *et al.*, 1989). There is only a single report of an *E. coli* strain engineered for lysine overproduction (Dauce-Le Reverend *et al.*, 1982). By combining a feedback-resistant aspartyl kinase with an increased level of dihydropicolinate synthase, lysine was produced at a level of 6.5 g/l.

Threonine

The first two steps in threonine biosynthesis are shared in common with that of lysine: side-chain phosphorylation of aspartate by aspartyl kinase followed by reduction to the semialdehyde (*Scheme 6*) (Cohen and Saint-Girons, 1987). The committed step to threonine is catalyzed by homoserine dehydrogenase, which catalyzes the reduction to homoserine. After phosphorylation, this is converted to threonine by threonine synthase. As noted above, *E. coli* has three aspartyl kinases that are each subject to end-product inhibition by lysine, threonine and methionine. *C. glutamicum* has only a single aspartyl kinase that is regulated by both threonine and lysine. Homoserine dehydrogenase and homoserine kinase are the other major control points for carbon flux; these are inhibited by threonine.

In contrast to the paucity of attempts to engineer lysine overproduction rationally,

Lysine

Dihydropicolinate synthase

Dihydropicolinate

Dihydropicolinate reductase

Tetrahydropicolinate

Tehydrohydropicolinyl succinylase

Succinyl diaminopimelate aminotransferase

Succinyl diaminopimelate

Succinyl diaminopimelate desuccinylase

L,L-Diaminopimelate

Diaminopimelate epimerase

meso-Diaminopimelate

meso-diaminopimelate dehydrogenase

CO_2

Lysine

Common pathway:

Aspartate → (ATP → ADP; *Aspartyl kinase*) → ^-O_2C–CH(NH_3^+)–CH_2–C(=O)–OPO_3^{-2} → (*Aspartate semialdehyde dehydrogenase*) → Aspartate semialdehyde

Methionine, Threonine, Isoleucine

Homoserine dehydrogenase → Homoserine

Homoserine → Methionine

Homoserine → (ATP → ADP; *Homoserine kinase*) → Homoserine phosphate → (*Threonine synthetase*) → Threonine

Threonine → (*Threonine deaminase*) → α-Ketobutyrate → (pyruvate → CO_2; *Acetohydroxy acid synthase*) → α-Aceto-α-hydroxybutyrate → (*Acetohydroxy acid isomeroreductase*) → α,β-Dihydroxy-β-methylvalerate → (*Dihydroxy acid dehydrase*) → α-Keto-β-methylvalerate → (*Transaminase*) → Isoleucine

Scheme 6

there have been numerous attempts to create threonine hyperproducers by using recombinant DNA techniques. Threonine overproduction is mainly dependent upon overcoming feedback regulation of aspartyl kinase and homoserine dehydrogenase. Such variants can be isolated by resistance to amino acid analogs by standard techniques. By overexpressing the threonine biosynthetic enzymes, strains that produce high levels of threonine can be produced. In many cases, production strains also had lesions in the isoleucine biosynthetic pathway in order to maximize threonine yields. Several groups have published very similar approaches to threonine overproduction, and their results are summarized in *Table 3* (Beppu, 1986; Miwa *et al.*, 1983; Shimizu *et al.*, 1995; Komatsubara, 1994; Reinscheid *et al.*, 1994; Ishida *et al.*, 1989, 1993; Moringa *et al.*, 1987; Nakamori *et al.*, 1987; Farfán *et al.*, 1996). Interestingly, while a feedback-resistant *E. coli* aspartyl kinase can be stably expressed at high levels in *C. glutamicum*, attempts to produce high levels of the corresponding *C. glutamicum* feedback-resistant enzyme in this host have met with frustration (Reinscheid *et al.*, 1994). This was finally overcome by using a cloning system that allowed only moderate overexpression of this enzyme. This is the major reason for the relatively low threonine yields from this strain, which was not optimized for amino acid overproduction. These authors speculate that these difficulties were due to the toxicity of accumulated threonine biosynthetic intermediates or their breakdown products. The use of baker's yeast is unusual for amino acid overproduction, although Farfán *et al.* (1996) also point out that the grown yeast cells can also be directly added to animal feeds, eliminating the costs of isolating the amino acid.

Isoleucine

As might be expected, isoleucine biosynthesis is tightly integrated with those of lysine, threonine and methionine (*Scheme 6*) (Unbarger, 1987). However, it is also intimately connected with valine production, since the enzymes catalyzing the final four steps of isoleucine biosynthesis also catalyze the analogous reactions that produce valine. The large number of steps involved and the connections with other pathways have made isoleucine one of the last amino acids targeted for overproduction. As a result, there are only a few examples of success in this area and the productivities of the strains reported are somewhat lower than those encountered in previous sections.

The first step in isoleucine production is the degradation of threonine into α-ketobutyrate by threonine deaminase. The activity of this enzyme is subject to feedback inhibition by the end product. After an aldol condensation with pyruvate, the α-hydroxyketone is rearranged and reduced to α,β-dihydroxy-β-methylvalerate. After conversion to the keto-isomer, the intermediate undergoes transamination to isoleucine. In the case of valine biosynthesis, the initial aldol condensation utilizes pyruvate as both the donor and acceptor; the remaining steps are analogous to those that lead to isoleucine.

The major issues in creating a strain that overproduces isoleucine are removing feedback inhibition of threonine deaminase and ensuring a sufficient supply of threonine (*Table 4*) (Komatsubara, 1994; Morbach *et al.*, 1995; Colón *et al.*, 1995). Komatsubara (1994) created an *S. marcescens* strain that combined feedback mutations in various enzymes along the entire pathway into a single strain. These mutations

Table 3. Production of threonine by engineered microbial strains

Strategy	Host	Yield (g/l)	Reference
Overexpressed *E. coli* threonine pathway enzymes; aspartyl kinase and homoserine dehydrogenase were feedback-resistant	*E. coli*	55	Beppu (1986)
Overexpressed *E. coli* threonine pathway enzymes in a host that overproduced threonine; aspartyl kinase and homoserine dehydrogenase were feedback-resistant	*E. coli*	65	Miwa *et al.*, (1983); Shimizu *et al.*, (1995)
Combined several feedback-resistant threonine pathway enzymes into a single strain; overexpressed threonine pathway enzymes from *S. marcescens* in this host; aspartyl kinase and homoserine dehydrogenase were completely feedback-resistant	*S. marcescens*	100	Komatsubara (1994)
Modestly overexpressed feedback-resistant variants of *C. glutamicum* aspartyl kinase and homoserine dehdrogenase; secretion was incapable of keeping up with production; cells also accumulated high levels of homoserine, isoleucine, glycine and lysine	*C. glutamicum*	8.2	Reinscheid *et al.*, (1994)
Overexpressed *E. coli* threonine pathway enzymes; aspartyl kinase and homoserine dehydrogenase were feedback-resistant	*C. glutamicum*	21	Beppu (1986)
Overexpressed *E. coli* threonine pathway enzymes; homoserine dehydrogenase was feedback-resistant; best results were obtained with a host that overproduced threonine	*C. flavum*	27	Ishida *et al.*, (1989)
Overexpressed feedback-resistant *C. lactofermentum* homoserine dehydrogenase and homoserine kinase in a strain that overproduced lysine	*C. lactofermentum*	33	Morinaga *et al.*, (1987); Nakamori *et al.*, (1987)
Overexpressed *E. coli* threonine pathway enzymes; used acetate as the carbon source	*C. flavum*	64	Ishida *et al.*, (1993)
Overexpressed feedback-resistant aspartyl kinase	*S. cerevisiae*	N.D.*	Farfán *et al.*, (1996)

*Threonine was not excreted from this strain; however, measurements of the intracellular concentration showed a 10-fold higher level in the recombinant strain as compared to the wild-type.

Table 4. Production of isoleucine by engineered microbial strains

Strategy	Host	Yield (g/l)	Reference
Combined a number of feedback-resistant mutations into a single strain; overexpressed the *S. marcescens* isoleucine pathway enzymes	*S. marcescens*	32	Komatsubara (1994)
Overexpressed a feedback-resistant *C. glutamicum* threonine deaminase in a strain that overproduced threonine	*C. glutamicum*	12.6	Morbach and Sahn (1995)
Overexpressed feedback-resistant *C. lactofermentum* homoserine dehydrogenase and homoserine kinase as well as the wild-type threonine deaminase from this organism	*C. lactofermentum*	15	Colón *et al.*, (1995)

were originally created by traditional strain breeding. This strain was further improved by overexpressing the *S. marcescens* isoleucine biosynthetic enzymes. Morbach *et al.* (1995) overexpressed a feedback-resistant *C. glutamicum* threonine deaminase in the strain that accumulated threonine described above (Reinscheid *et al.*, 1994) to achieve reasonable isoleucine overproduction. A similar strategy was reported by Colón *et al.* (1995) using *C. lactofermentum.*

Proline

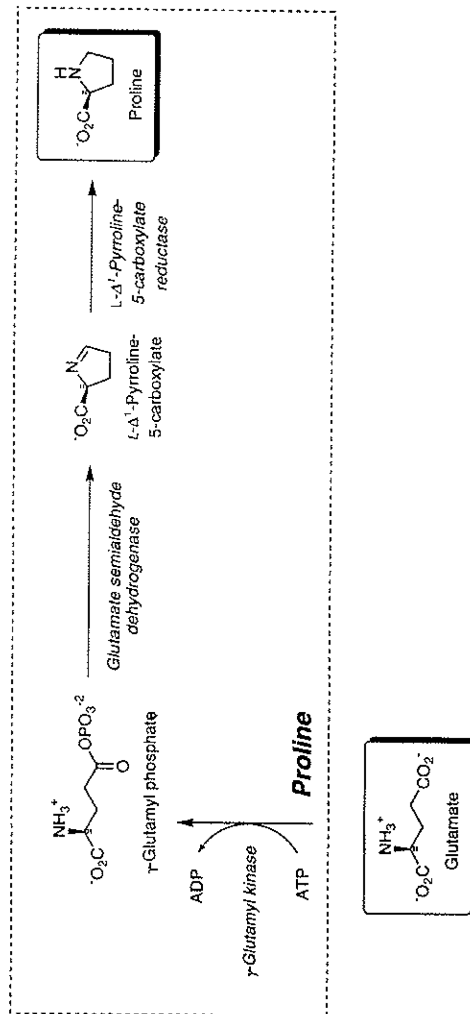
Proline is synthesized in three steps from L-glutamate (*Scheme 7*, top) (Leisinger, 1987). The side-chain carboxylate is phosphorylated by γ-glutamyl kinase, then this is reduced to the aldehyde by glutamate semialdehyde dehydrogenase. Cyclization to L-Δ^1-pyrroline-5-carboxylate occurs spontaneously and this is subsequently reduced to proline in the final step. As expected, the major regulatory point is feedback inhibition of γ-glutamyl kinase by proline.

There has been relatively little work on proline overproduction by recombinant microorganisms, largely due to the success of classical strain breeding (Yoshinaga, 1986). However, three examples have been reported that utilize *E. coli, S. marcescens* or *C. glutamicum* as the host cells (*Table 5*) (Beppu, 1986; Komatsubara, 1994; Yoshinaga, 1986). In each case, the proline biosynthetic enzymes were overproduced. One noteworthy aspect of the overproduction by *S. marcescens* was that a selection step for tolerating high osmolarity was required to achieve proline hyperproduction (Komatsubara, 1994).

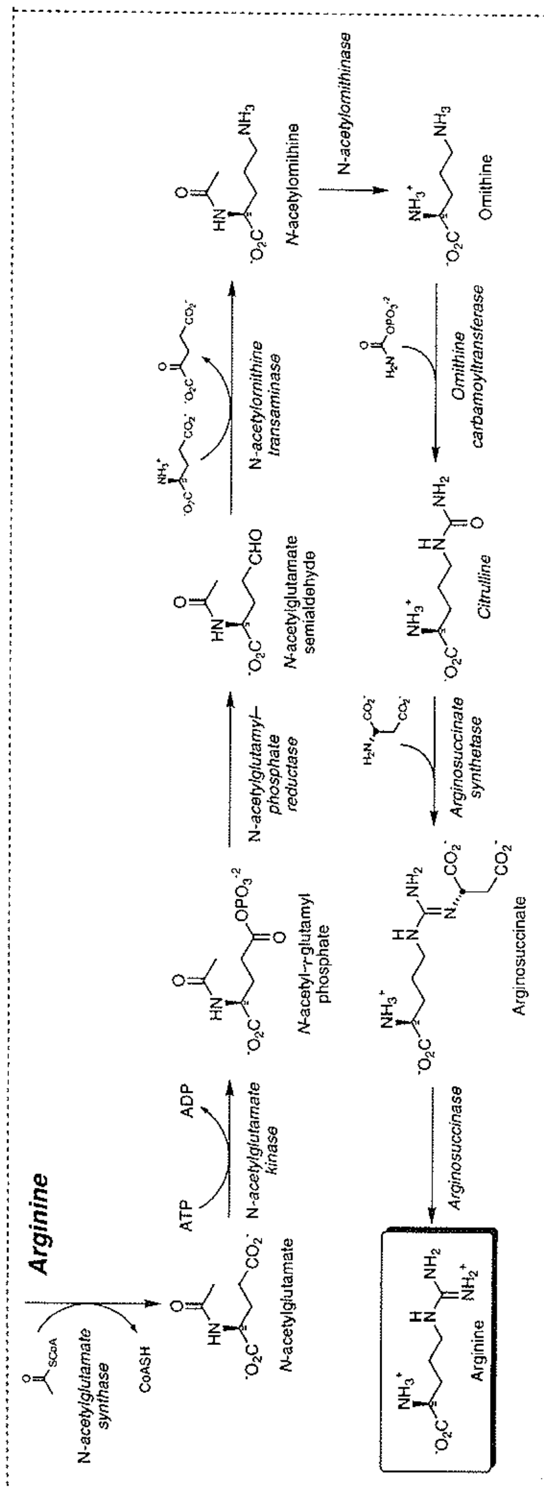
Arginine

Arginine biosynthesis is intimately associated with that of polyamines and with the urea cycle (Glansdorff, 1987). As a result of its length and complexity, only a few studies on creating overproducing strains by recombinant DNA techniques have appeared. The first committed step is the acetylation of glutamate catalyzed by *N*-acetylglutamate synthase (*Scheme 7*, bottom). This is followed by phosphorylation of the side-chain carboxylate and its reduction to an aldehyde. Note the resemblance between this pathway and that of proline biosynthesis; the major difference being that acetylation of the α-amine prevents cyclization of the aldehyde intermediate in this case. After reductive amination to form ornithine, the acetyl protecting group is removed and the side-chain urea is constructed by reaction with carbamoyl phosphate. The final nitrogen is derived from the α-amino group of aspartate. The final intermediate is converted to arginine by arginosuccinase.

In *E. coli*, feedback regulation of the first step of the pathway (*N*-acetylglutamate synthase) is the major control point. In *C. glutamicum*, the second step is also subject to feedback inhibition. Momose *et al.* created an *E. coli* overproduction strain for arginine by overexpressing a feedback-resistant mutant of *N*-acetylglutamate synthase in an *E. coli* host that had been selected by classical breeding techniques. The recombinant organism produced arginine at a level of 19 g/l (Yoshida, 1986). A somewhat different approach was used to engineer an *S. marcescens* strain that produced 40 g/l of arginine after removal of feedback regulation and disabling the enzymes responsible for arginine degradation (Komatsubara, 1994).



Proline
Glutamate
ATP
ADP
γ-Glutamyl kinase
γ-Glutamyl phosphate
Glutamate semialdehyde dehydrogenase
L-Δ1-Pyrroline-5-carboxylate
L-Δ1-Pyrroline-5-carboxylate reductase
Proline



Scheme 7

Table 5. Production of proline by engineered microbial strains

Strategy	Host	Yield (g/l)	Reference
Overexpressed the *E. coli* proline biosynthetic enzymes; added L-glutamate to the growth medium	*E. coli*	14	Yoshinaga (1986)
Used strain breeding and combined beneficial mutations into a single strain that could tolerate high osmolarity; overexpressed feedback-resistant *S. marcescens* γ-glutamyl kinase and glutamate semialdehyde dehydrogenase	*S. marcescens*	100	Komatsubara (1994)
Overexpressed the *E. coli* proline biosynthetic enzymes	*C. glutamicum*	16	Beppu (1986)

OVERPRODUCTION OF OTHER NATURAL PRODUCTS BY RECOMBINANT MICROBES

In addition to using metabolic engineering to overproduce amino acids, similar approaches have also been used to create strains that produce other valuable natural products. Examples in which metabolite production has been increased by recombinant DNA methods are summarized in *Table 6* (Shiomi *et al.*, 1988, 1995; Murata, 1994; Hagihara *et al.*, 1995; Compagno *et al.*, 1993; Porro *et al.*, 1995; Ensley *et al.*, 1983; della-Cioppa *et al.*, 1990, Yamano *et al.*, 1994; Anderson *et al.*, 1985). In many cases, the organisms used for production are not those in which the metabolic pathway was originally found. This is a key advantage of the recombinant DNA approach, allowing one to choose an organism for its suitability for large-scale growth and production instead of being forced to improve a naturally occurring organism that may have undesirable properties (slow growth, pathogenicity, etc.). In general, the yields of these products are much lower than those of the amino acids that were discussed in the previous section. This is due to the more complex biosynthetic pathways involved in these examples as well as the nature of the projects, many of which were intended as demonstrations rather than industrial processes. Three cases in this section also illustrate another method for using engineered microbes in chemical production: cells that express enzymes of interest are grown and harvested, their membranes are permeabilized and then substrates and cofactors are added to the system. In this approach, the recombinant cells act as synthetic equivalents of a purified enzyme, but in a much simpler form.

S-Adenosylmethionine

S-Adenosylmethionine is currently produced by extraction from yeast cells that are cultured in the presence of methionine (Shiozaki *et al.*, 1984). This key intermediate in C_1 metabolism is produced by methionine adenosyltransferase (*Scheme 8*). Shiomi and co-workers have created a strain of *S. cerevisiae* that overexpresses this enzyme and accumulated 0.5 g of *S*-adenosylmethionine per gram of dry cells when the medium was supplemented with methionine (Shiomi *et al.*, 1988, 1995). This was approximately 20-fold higher production than was achieved with the unmodified cells. Unfortunately, the large size of *S*-adenosylmethionine precludes its secretion from the yeast cells.

Glutathione

Glutathione is a commercially-important tripeptide that is synthesized by two enzymes, glutathione synthetase A and B (*Scheme 9*). Glutathione synthetase A is subject to feedback inhibition by the end product. The present industrial methods for its production are extraction from yeast cells or chemical synthesis; both of these processes are costly and time-consuming (Murata, 1994). Murata and co-workers have shown how metabolic engineering can be used to overcome problems with both overproducing and isolating a natural metabolite (Shiomi *et al.*, 1995; Murata, 1994; Murata and Kimura, 1982; Murata *et al.*, 1983). Because of its size and charge, secretion of glutathione from growing cells was considered unlikely. Therefore, a slightly different strategy was used: *E. coli* cells were engineered to overexpress

Table 6. Overproduction of natural metabolites by engineered microbial strains

Product	Strategy	Host	Yield	Reference
S-adenosylmethionine	Overexpressed *S. cerevisiae* methionine adenosyltransferase	*S. cerevisiae*	0.50 g/g dry cells	Shiomi *et al.*, (1988, 1995)
Glutathione	Overexpressed feedback-resistant glutathione synthetase A and wild-type glutathione synthetase B from *E. coli*; immobilized cells were used and substrates and cofactors were supplied exogenously	*E. coli*	25 g/l	Murata (1994)
FAD	Overexpressed *C. ammoniagenes* FAD synthetase; after cell growth and permeabilization, substrate and cofactors were added exogenously	*Corynebacterium ammoniagenes*	12.7 g/l	Hagihara *et al.*, (1995)
Fructose-1,6-diphosphate	Overexpressed *E. coli* β-galactosidase; after growing, yeast cells were permeabilized and used directly for the reaction	*S. cerevisiae*	32 g/l	Compagno *et al.*, (1993)
L-(*S*)-Lactate	Overexpressed bovine muscle lactate dehydrogenase; used a sophisticated fermentation method for best production	*S. cerevisiae*	20 g/l	Porro *et al.*, (1995)
Indigo	Overexpressed *Pseudomonas putida* napthalene dioxygenase	*E. coli*	0.025 g/l	Ensley *et al.*, (1983)
Melanin	Overexpressed *Streptomyces antibioticus* tyrosinase proteins; culture was supplied with Cu(II) and tyrosine	*E. coli*	Not determined	della-Cioppa *et al.*, (1990)
β-Carotene	Expressed four β-carotene biosynthetic enzymes from *Erwinia uredovora*	*S. cerevisiae*	0.002 g/g dry cells	Yamano *et al.*, (1994)
2-Keto-L-gulonate	Expressed *Corynebacterium sp.* 2,5-diketo-D-gluconic acid reductase	*Erwinia herbicola*	1 g/l	Anderson *et al.*, (1985)
2-Keto-L-gulonate	Overexpressed *Corynebacterium sp.* 2,5-diketo-D-gluconic acid reductase; used strain improvement to avoid overmetabolism of the desired product	*Erwinia citreus*	20 g/l	Grindley *et al.*, (1988)

Methionine + Adenosine triphosphate (ATP) → (*Methionine adenosyltransferase*) → *S*-adenosylmethionine

Scheme 8

$^{-}O_2C$–CH(NH_3^+)–CH$_2$CH$_2$–CO_2^- + $^{-}O_2C$–CH(NH_3^+)–CH$_2$SH → (ATP → ADP + P_i; *Glutathione synthetase A*) → γ-*L*-Glutamyl-*L*-cysteine

γ-*L*-Glutamyl-*L*-cysteine + $^{+}H_3N$–CH$_2$–CO_2^- → (ATP → ADP + P_i; *Glutathione synthetase B*) → γ-*L*-Glutamyl-*L*-cysteinylglycine (Glutathione)

Scheme 9

glutathione synthetases A and B in which the former was resistant to feedback inhibition. After the cells had grown, they were harvested, immobilized in a κ-carageenan gel matrix and permeabilized by partially dissolving their membranes with toluene. Glutathione was then produced by adding a solution of the amino acid precursors and ATP. Under optimized conditions, the system produced glutathione at a level of 25 g/l (Murata, 1994). Thus, by immobilizing and only partially permeabilizing the cells, they acted as the synthetic equivalent of the purified synthetase enzymes.

Flavin Adenine Dinucleotide (FAD)

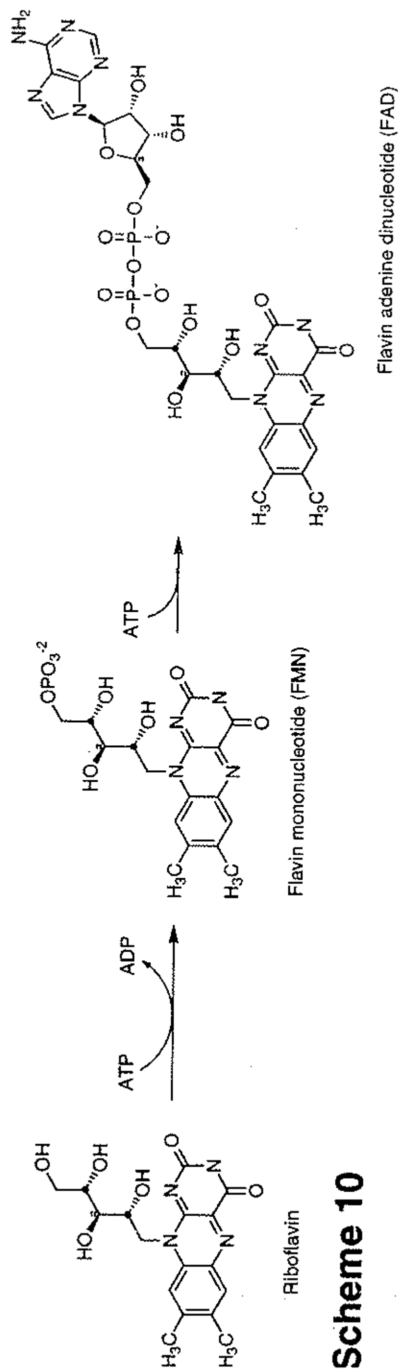
Flavins are used as nutritional supplements; however, industrial methods for their production are not well-developed. The problems with large-scale flavin production are similar to those discussed in connection with glutathione. The difficulties involved in product isolation and secretion suggested that reactions with permeabilized cells might again be the most fruitful approach. Therefore, to create a strain for overproducing flavin adenine dinucleotide (FAD), Hagihara and co-workers (1995) overexpressed the bifunctional flavokinase/FMN adenyltransferase from *Corynebacterium ammoniagenes* in the same bacterium (*Scheme 10*). Once the recombinant cells had grown, their membranes were partially dissolved by adding xylene and riboflavin and ATP were added to the suspension. Using this strategy, a 94% yield of FAD was obtained.

Fructose-1,6-diphosphate

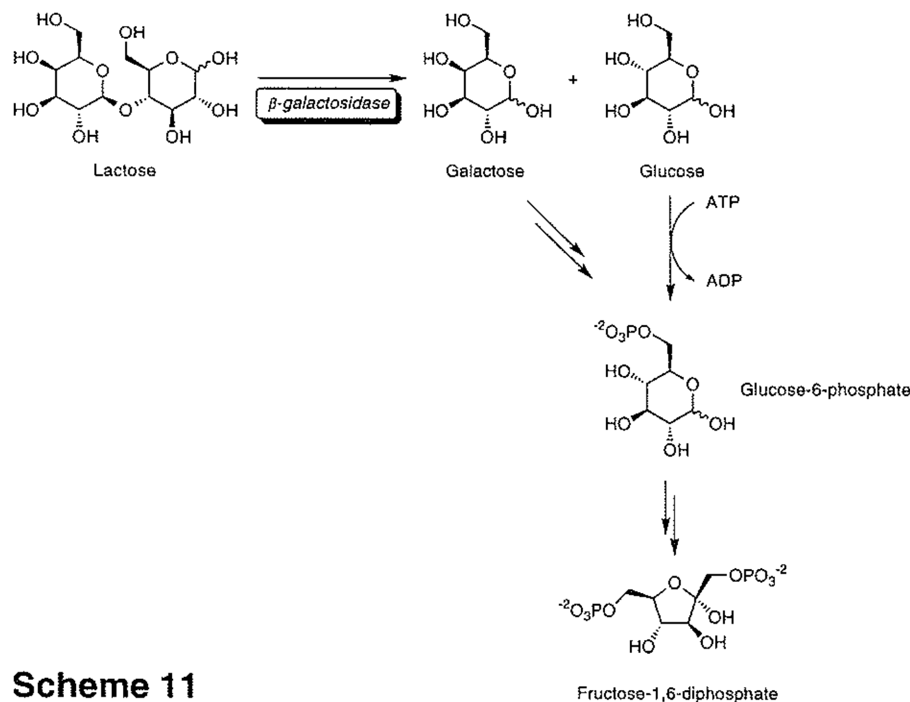
Creating value-added products from waste materials is a key goal in biotechnology. Cheese whey is the major byproduct of the dairy industry and its disposal is becoming increasingly costly (Irvine and Hill, 1985). After most of the protein and other useful components have been removed, the major component is lactose, a product with little commercial value (Porro *et al.*, 1992). This situation has motivated efforts to convert lactose into products that can be sold for a profit. Fructose-1,6-diphosphate (FDP) is a key intermediate in glycolysis (*Scheme 4*) and there is increasing industrial interest in its production. Compagno *et al.* (1993) developed a method for converting the lactose found in whey into FDP by overexpressing *E. coli* β-galactosidase in baker's yeast. Wild-type baker's yeast is unable to cleave the glycosidic bond of lactose; however by expressing the *E. coli* enzyme, grown yeast cells could convert the liberated glucose and galactose into fructose-1,6-diphosphate at a level of 32 g/l (*Scheme 11*). By altering the growth conditions of the recombinant yeast, it was possible to isolate the galactose liberated from lactose or to convert both the glucose and the galactose into fructose-1,6-diphosphate. To provide access to the cellular enzymes, the yeast cells were permeabilized with toluene before lactose was added. As in the previous two cases, this arrangement simplified product isolation.

L-(S)-Lactate

Lactic acid is an industrially important product that is generally obtained by fermentation using various *Lactobacillus* species. The use of these organisms complicates the



Scheme 10



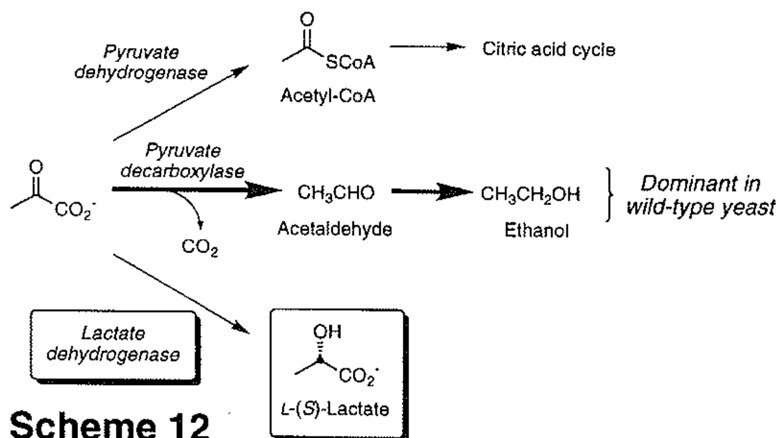
Scheme 11

process, however, since they are acid-intolerant. This requires that the pH of the fermentation mixture be kept around 5; however, the solubility of lactic acid at this pH value is relatively low and the fermentation mixtures solidify, complicating product isolation. To overcome these difficulties, Porro and co-workers (1995) engineered baker's yeast to produce lactic acid. Yeast was chosen for its acid-tolerant growth and the long experience with this organism in the food industry.

There are two major pathways for carbon assimilation in baker's yeast that differ in the fate of pyruvate (the end product of glycolysis). The first, discussed above (*Scheme 4*), is its oxidative decarboxylation to acetyl-CoA, which enters the citric acid cycle (*Scheme 12*, top). However, in fermenting yeast, the major pathway involves decarboxylation by pyruvate decarboxylase to form acetaldehyde, which is subsequently reduced to ethanol (*Scheme 12*, middle). To produce lactate from pyruvate, Porro and co-workers (1995) expressed bovine muscle lactate dehydrogenase (*Scheme 12*, bottom). As anticipated, the major difficulty was competition between lactate dehydrogenase and pyruvate decarboxylase for the pyruvate pool. This was solved by a clever two-stage fermentation system that minimized ethanol production and provided lactate at a level of 20 g/l. Unfortunately, this yield is still five-fold below current processes based on *Lactobacilli*, although the authors point out that their approach dramatically simplifies product isolation.

Indigo

During the cloning of dioxygenase genes from *Pseudomonas putida* strains that



Scheme 12

degrade aromatics in *E. coli*, it was noticed that some of the recombinant cultures produced a dark blue precipitate that was subsequently identified as indigo (Ensley *et al.*, 1983). Several lines of evidence implicated tryptophanase as the source of indole, and the pathway shown in *Scheme 13* was proposed. While the level of indigo produced was rather low, the interplay between a host enzyme, a cloned gene and spontaneous chemistry is remarkable.

Melanin

There has been recent interest in melanins for several industrial applications. These black, insoluble pigments are formed by the oxidation of tyrosine to the corresponding quinone, which then undergoes polymerization (*Scheme 14*). To create an *E. coli* strain that synthesized melanin, dell-Cioppa *et al.* (1990) expressed two *Streptomyces* biosynthetic enzymes and supplemented the culture with tyrosine and Cu(II). Unfortunately, the melanin produced was not isolated and quantitated so its yield is unknown.

β-Carotene

To show that a complete biosynthetic pathway for a secondary metabolite could be transferred into a eukaryotic cell, Misawa and co-workers (Yamano *et al.*, 1994) expressed four proteins catalyzing β-carotene synthesis in baker's yeast. This tetraterpene is derived from farnesyl and isopentenyl pyrophosphate, which are intermediates in yeast sterol biosynthesis (*Scheme 15*). While the overall yield of β-carotene was relatively low, this system represents a first-generation attempt to transfer an entire metabolic pathway from one organism to another.

2-Keto-L-gulonate

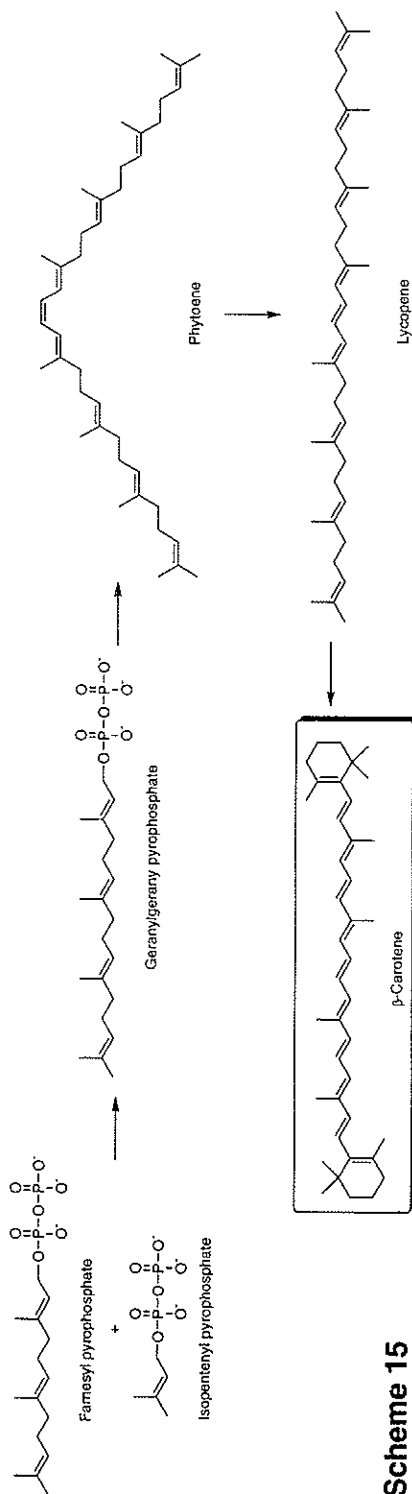
The Reichstein-Grussner synthesis (Reichstein and Grössner, 1934) is the

L-tryptophan → (Tryptophanase; − Serine) → Indole → (Napthalene dioxygenase) → cis-diol → (Spontaneous) → Indoxyl → (Air) → Indigo

Scheme 13

Tyrosine → (Tyrosinase) → [DOPA] → (Tyrosinase) → [dopaquinone] → Melanin

Scheme 14



Scheme 15

classical route to ascorbic acid (*Scheme 16*, top). Its major deficiency is the need for a protection/deprotection scheme that allows the selective oxidation of one hydroxyl to the carboxylic acid. Because enzymes have much higher selectivities than typical organic reagents, a fermentation route was sought in order to simplify this process. In the most highly-optimized case, Sonoyama *et al.* reported that co-fermentation of an *Erwinia* and a *Corynebacterium* species afforded a direct synthesis of 2-keto-L-gulonic acid (2-KLG) from glucose (Sonoyama *et al.*, 1975, 1976; Kita and Hall, 1981). This intermediate can be easily converted to ascorbate by heating in the presence of acid (*Scheme 16*, middle). To simplify this process even further, an *Erwinia herbicola* and an *Erwinia citreus* species capable of converting glucose to 2,5-diketo-L-gulonic acid (2,5-DKG) were engineered to express a *Corynebacterium* reductase responsible for reduction of 2,5-DKG to 2-KLG (Anderson *et al.*, 1985; Grindley *et al.*, 1988). Thus, these engineered organisms condensed five steps of a conventional chemical synthesis into a single fermentation. Despite its elegance, this one-step fermentation process has not displaced the Reichstein-Grussner route on a commercial scale for economic reasons discussed in detail by Turner (1995).

SYNTHESIS OF ANTIBIOTICS BY RECOMBINANT MICROBES

The increasing number of bacterial species that are resistant to antibiotics is becoming a serious health issue (Spratt, 1994; Davies, 1994). This trend has spurred efforts to increase the pace at which new antibiotics can be developed and brought to the clinic. While much attention has been focused on chemical routes to novel antibiotics, several groups are also exploring the use of recombinant microorganisms to help solve these problems. Some recent efforts in this area are summarized below.

Polyketides

Polyketides produced by bacteria and fungi continue to dominate screens of natural products for biological activity. This is due to their rich diversity of structure and widespread occurrence. While traditional screening remains an important method for discovering new structures, several groups have combined biosynthetic studies with molecular biology to unravel the rules by which polyketides are assembled. The long-term goal is to create novel structures to order by genetic engineering. While this is not possible at the present, good progress has been made and some successful examples are discussed below (Simpson, 1995). It should be realized, however, that none of these engineered polyketides have been produced at levels sufficient for scale-up.

Polyketide biosynthesis is closely related to fatty acid biosynthesis (*Scheme 17*). In this pathway, a growing carbon chain is linked to a carrier protein by a thioester linkage. A malonyl thioester is enzymatically decarboxylated and condensed with the growing chain in a Claisen condensation. This extends the chain by two carbons and results in the formation of a β-keto thioester. The ketone is reduced to the corresponding alkane by a three-step process involving reduction to the alcohol, dehydration to an olefin and reduction of the enoyl thioester. This cycle continues until the chain reaches a pre-set length (typically 16 or 18 carbons), when a thioesterase liberates the free fatty acid. In polyketide biosynthesis, variations on this theme are introduced by the omission of one or more steps in the reduction of the β-keto thioester intermediate

and by the incorporation of propionate or butyrate units in place of one or more acetate moieties. These alterations provide for enormous structural diversity.

There are two general classes of polyketides: macrolides, exemplified by erythromycin and rapamycin, and aromatic polyketides such as tetracycline and daunomycin. While both varieties are produced from polyketide precursors, their genetic and biochemical organizations are different. These differences turn out to have a large impact on engineering polyketide biosynthesis.

Most work in re-engineering polyketide biosynthesis has centered on various *Streptomycetes*. In addition to being natural producers of polyketide antibiotics, studies of these filamentous bacteria also have the advantage of well-developed genetics and cloning methodology. Once genes encoding biosynthetic enzymes in these pathways had been cloned, it was quickly apparent that the genetic organization mirrored the biosynthetic pathway (Donadio *et al.*, 1991). Inserting these genes into non-antibiotic-producing strains of *Streptomyces* conferred the ability to produce the polyketide, demonstrating that these gene clusters encoded all the necessary biosynthetic information. Once this had been shown, the next logical step was to re-engineer a biosynthetic pathway by combining enzymes from two different pathways in the same organism. The first successful examples of this strategy were reported in 1985 (Hopwood *et al.*, 1985) when the enzymes responsible for the biosynthesis of actinorhodin were introduced into strains of *Streptomyces* that synthesized either medcrimycin or granaticin (*Scheme 18*, top). In both cases, new polyketides (mederrhodin A and dihydrogranatirhodin) were produced that combined aspects of both pathways. While this experiment proved that 'hybrid' antibiotics could be produced by combining polyketide biosynthetic genes, no attempt was made to rationally control their structure or to optimize their production level.

Rational redesign of polyketide biosynthesis has proved a more elusive goal that requires a deeper understanding of the genetic and biochemical aspects of the pathway. In macrolide biosynthesis, there appears to be a one-to-one correspondence between enzyme active sites and biosynthetic steps. The active sites are generally organized on large proteins with ten or more active sites that catalyze successive steps in a pathway. Furthermore, the genetic arrangement mirrors the biochemistry, with genes encoding enzymes catalyzing 'early' steps located upstream from those encoding 'late' enzymes. Using this apparently simple code that links the genetics of the system with the chemical steps, Katz and co-workers (Donadio *et al.*, 1993) altered the biosynthesis of 6-deoxyerythronolide B to incorporate a C_6,C_7 double bond (*Scheme 18*, bottom). Based on the genetic organization, it was expected that this modification could be introduced by inactivating the enoyl reductase that occurs near the end of the second protein. The enoyl reductase gene was modified to encode an inactive protein, then the modified biosynthetic genes were introduced into a non-antibiotic-producing *Streptomyces* host. The major polyketide produced by this recombinant organism was identified as $\Delta^{6,7}$-anhydroerythromycin C, which incorporated the expected unsaturation. The additional hydroxyl group at C_{12} was unexpected, but also precedented from other erythromycins. This result, along with two other examples from the same group (Donadio *et al.*, 1991; Kao *et al.*, 1994), showed the feasibility of rationally re-engineering macrolide polyketides.

In contrast to macrolide biosynthesis, the enzymes involved in aromatic polyketide synthesis use a single set of active sites in an iterative fashion to build up the polyketide

Reichstein & Grussner, 1934:

D-glucose

H_2

D-Sorbitol

Acetobacter suboxydans

L-Sorbose

H^+,

Diacetone-L-sorbose

[O]

Diacetone-2-keto
L-gulonic acid

H^+

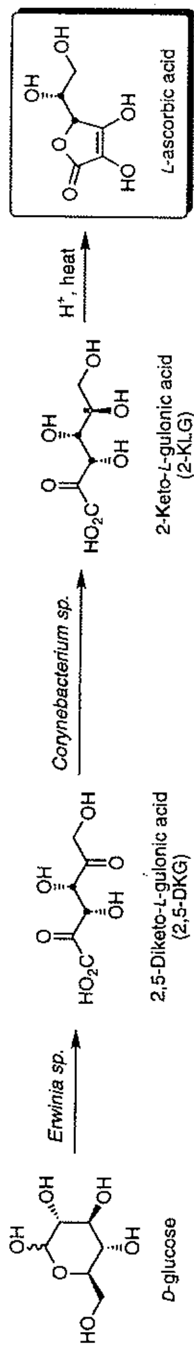
2-Keto-L-gulonic acid
(2-KLG)

H^+, heat

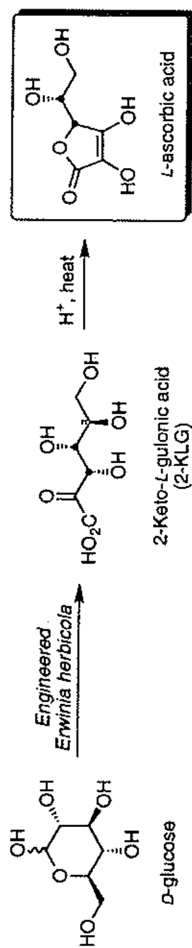
L-ascorbic acid

Scheme 18

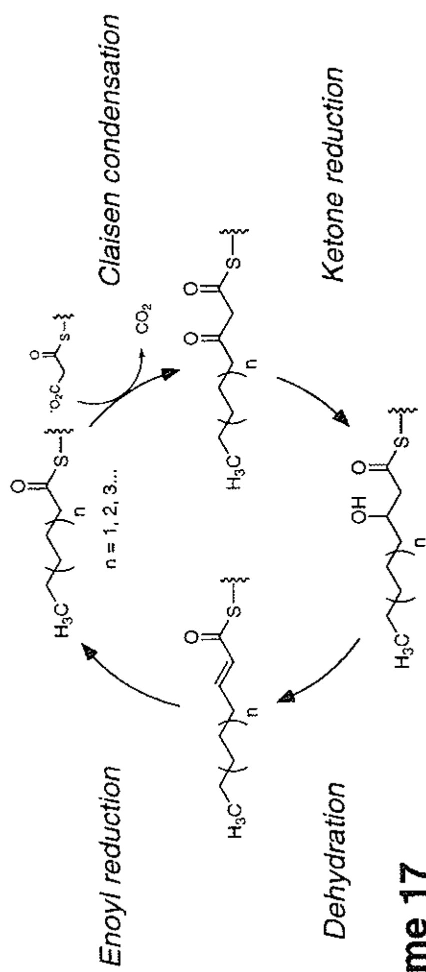
Sonoyama et al., 1982:



Anderson et al., 1985:



Scheme 16



Scheme 17

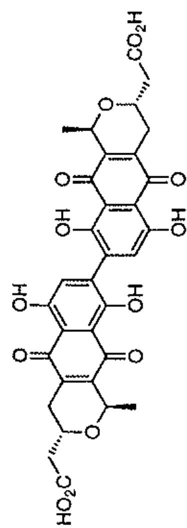
skeleton. For this reason, it is not possible to predict the structure of an aromatic polyketide from the genetic organization alone. The overall picture that has emerged from biosynthetic studies is that a 'minimal polyketide synthase' is responsible for creating what can be at least formally drawn as a straight-chain polyketide and that other, separate enzymes are responsible for subsequent reductions, cyclizations and aromatizations (McDaniel *et al.*, 1993). Thus, rationally re-engineering aromatic polyketide biosynthesis requires pairing a 'minimal polyketide synthase' with appropriate downstream processing enzymes. In an elegant demonstration of this approach, Khosla and co-workers showed how the proper choice of ketone reductase and aromatase could yield a new polyketide whose structure had been correctly predicted. The tetracenomycin minimal polyketide synthase creates a 20-carbon polyketide skeleton and catalyzes the initial cyclization between C_7 and C_{12} *if there is a C9 ketone*. A series of non-enzyme-catalyzed steps leads to two polyketides (SEK 15 and SEK 15b) in approximately equal amounts (*Scheme 19*) (McDaniel *et al.*, 1994). Intermediates shown in this scheme in parentheses are hypothetical; they have not been isolated. By introducing the ketone reductase from the actinorhodin biosynthetic pathway (which reduces C9 ketones in such polyketides) as well as the aromatase from the griseusin pathway (which catalyzes the formation of an aromatic ring from C_7 – C_{12}), the major polyketide was identified as SEK 43 (McDaniel *et al.*, 1995). This structure could be predicted on the basis of the introduced enzyme activities and shows that even in the complex case of aromatic polyketide biosynthesis, rational engineering is possible.

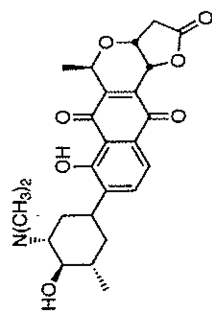
β-*Lactams*

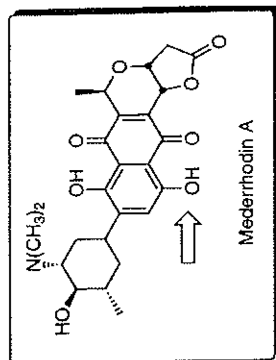
Semi-synthesis has proved remarkably effective in generating new β-lactam antibiotics that evade bacterial resistance to this class of pharmaceuticals. This strategy requires efficient access to the penicillin and cephalosporin nuclei, and a great deal of classical strain breeding has been used to create strains of *Penicillium chrysogenum* and *Acremonium chrysogenum* that are useful in industrial-scale production of penicillin G and cephalosporin C, respectively (Vichitsoonthonkul *et al.*, 1994). The enzymatic pathways for the biosynthesis of these β-lactams have been elucidated (*Scheme 20*) (Skatrud, 1992). In both cases, a linear tripeptide is oxidatively cyclized to isopenicillin N. In penicillin biosynthesis, the side-chain amide is cleaved by to yield free 6-aminopenicillinic acid which is acylated by phenylacetyl-CoA to afford penicillin G (benzyl penicillin) (*Scheme 20*, top). In the formation of cephalosporin C, the side-chain of isopenicillin N is first epimerized to the D-configuration, then an oxidative ring expansion occurs to produce deacetoxycephalosporin C (DAOC). In *A. chrysogenum*, the expandase enzyme also catalyzes a further oxidation of DAOC to deacetylcephalosporin C (DAC). Acetylation of the free hydroxyl by acetyl-CoA completes the biosynthesis of cephalosporin C (*Scheme 20*, bottom).

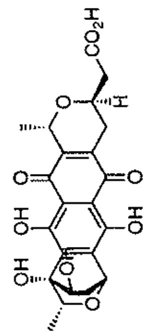
The industrial synthesis of clinically-useful cephalosporins currently involves chemical ring expansion of a penicillin. Chemical ring expansion is a complex process that adds several steps to these syntheses and requires large amounts of organic solvents. Recombinant DNA techniques have been used to improve direct fermentation routes to cephalosporins and to remove the need for chemical ring expansion. One strategy has been to increase the level of cephalosporin C production by *A. chrysogenum*.

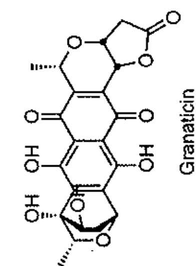
Hopwood et al., 1985:

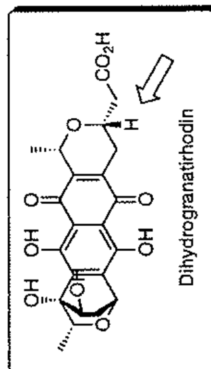

Actinorhodin


Medermycin

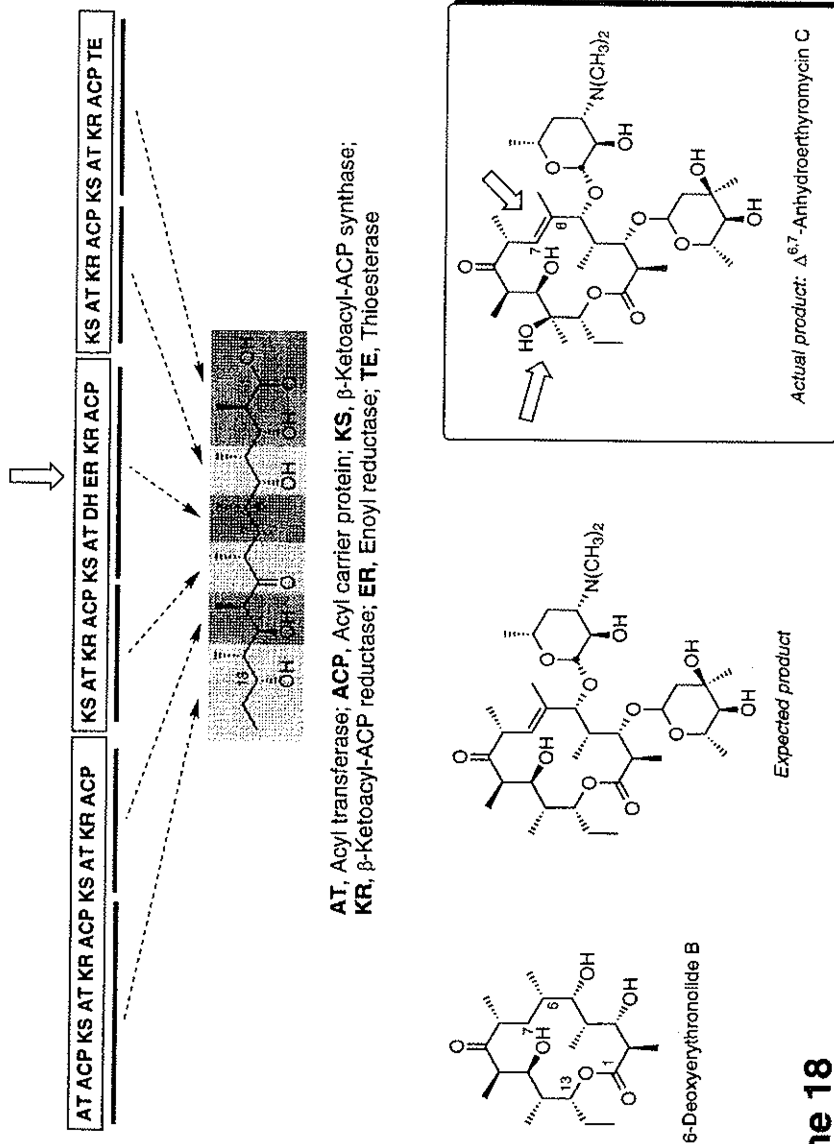

Mederrhodin A


Dihydrogranaticin

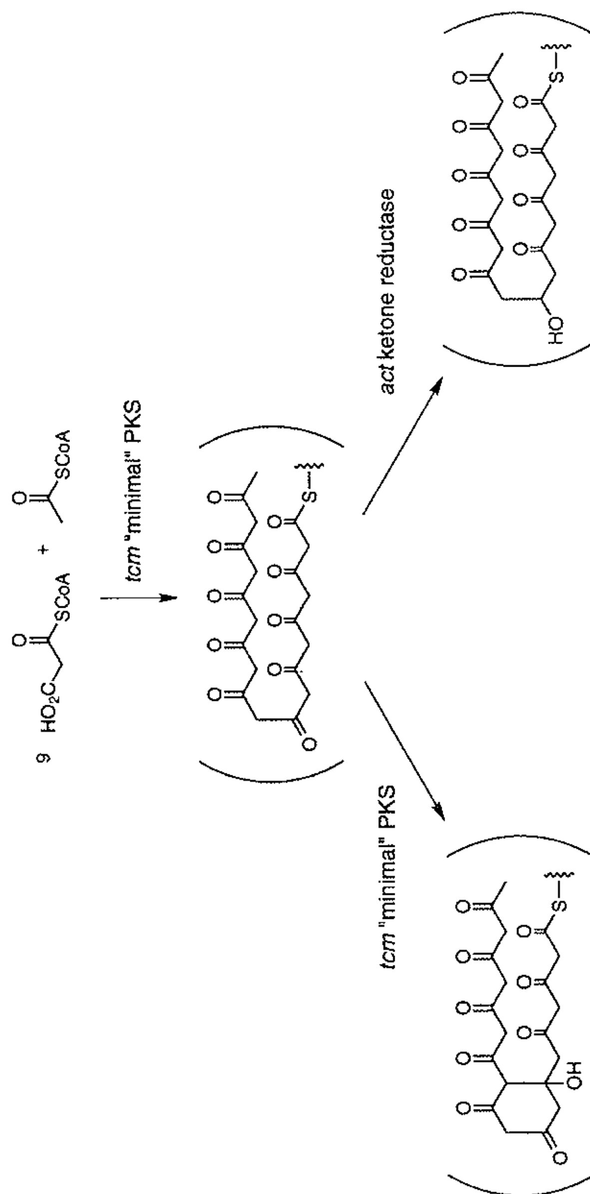

Granaticin


Dihydrogranatirhodin

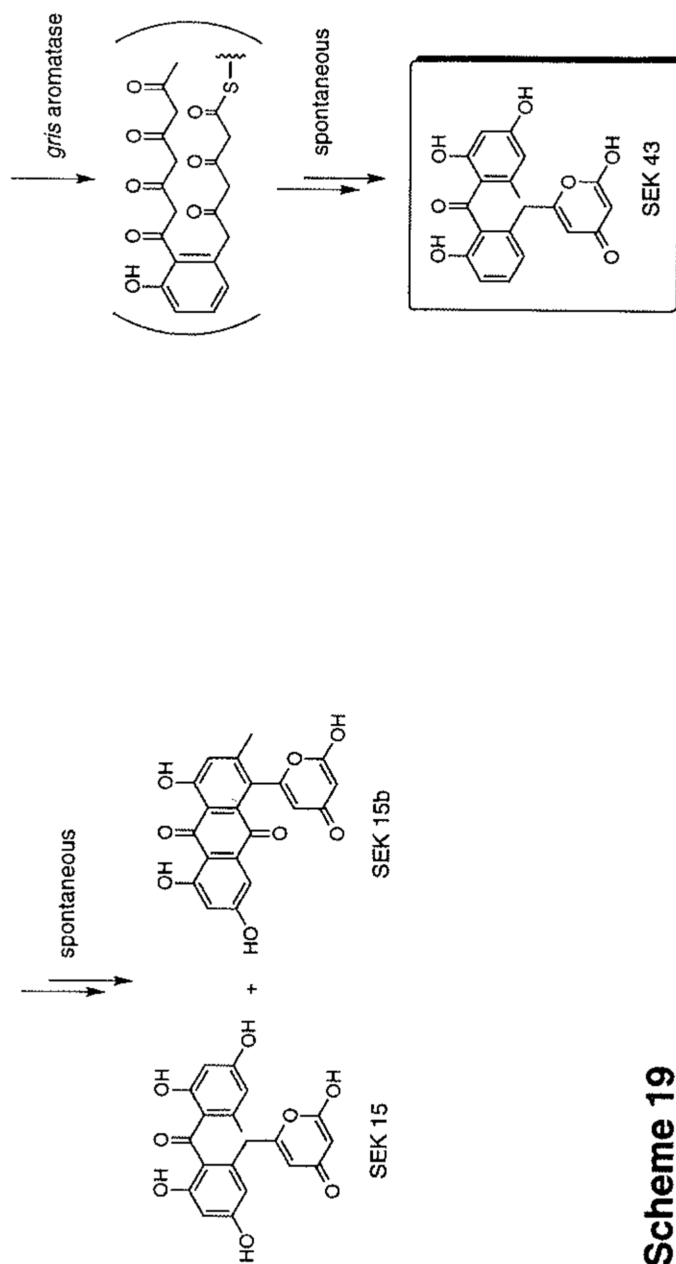
Donadio et al., 1993:



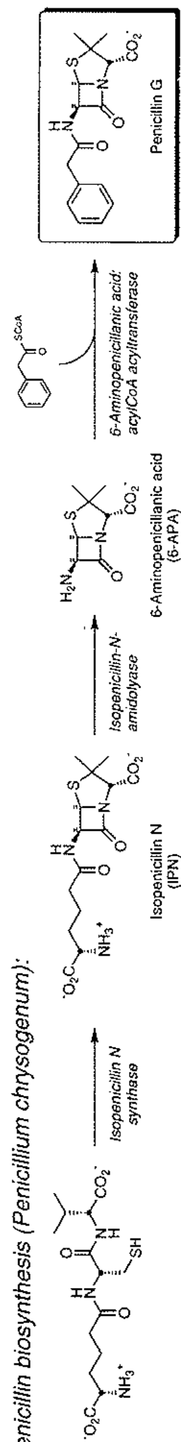
Scheme 18

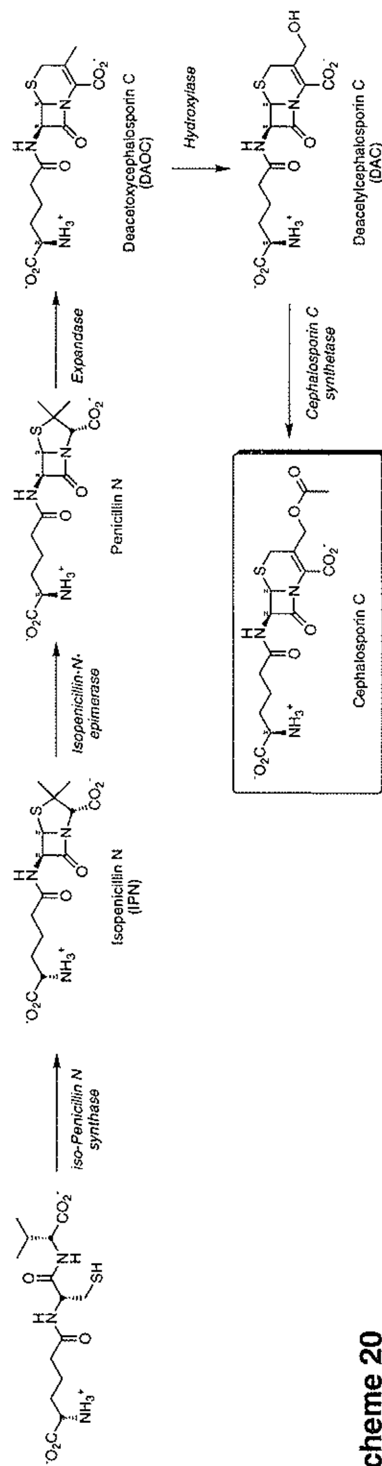


9
SCoA
+
SCoA
tcm "minimal" PKS
act ketone reductase
tcm "minimal" PKS



Scheme 19



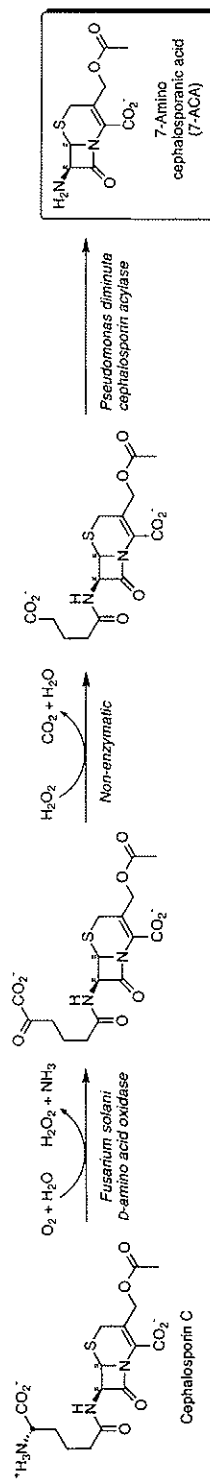


Scheme 20

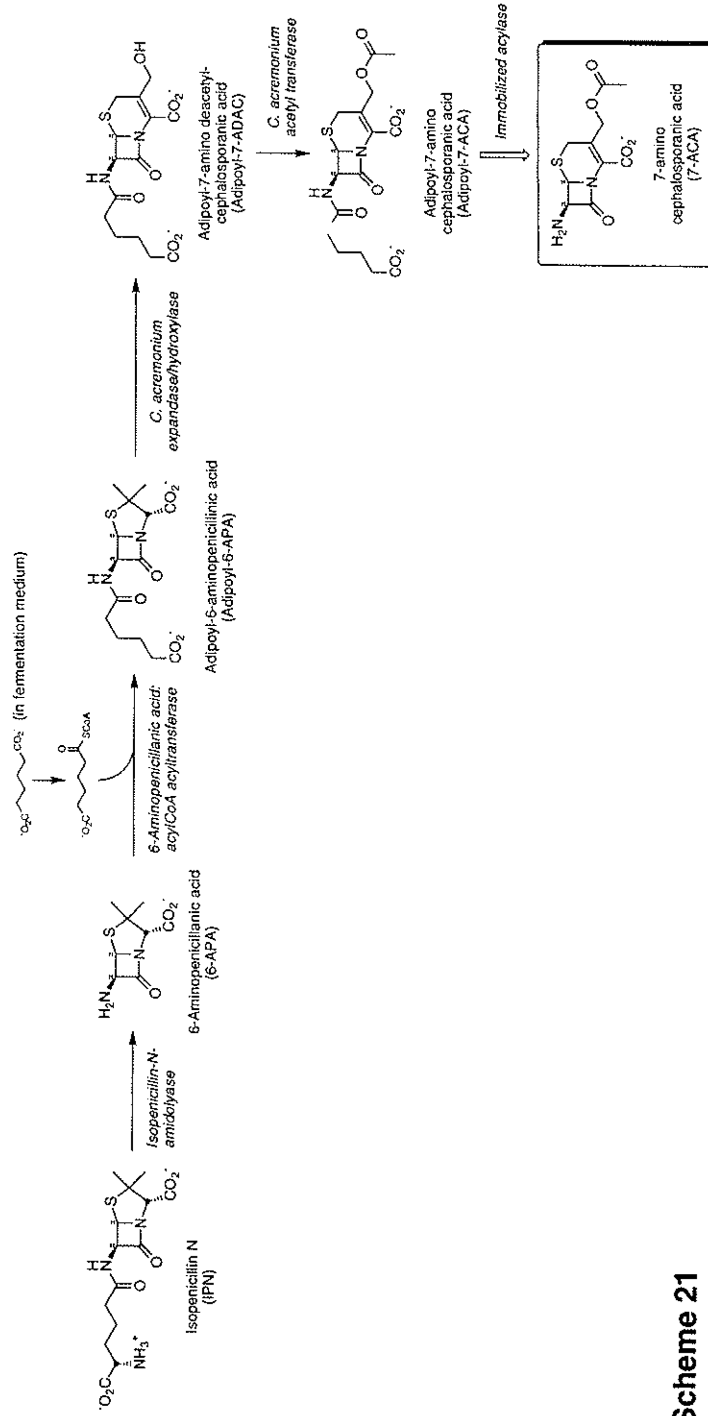
In one approach, Queener and co-workers overexpressed the *A. chrysogenum* expandase in this host (Skatrud *et al.,* 1989). This enzyme was chosen since the existing strain accumulated penicillin N in addition to cephalosporin C, suggesting that expandase activity was insufficient for the carbon flux through the pathway (*Scheme 20*). In pilot-scale fermentations, expandase overexpression improved production of cephalosporin C by 15%. In another approach, Hughes and co-workers (DeModena *et al.,* 1993) obtained a two-fold increase in cephalosporin C production by overexpressing a bacterial hemoglobin in *A. chrysogenum*. Three steps in cephalosporin C biosynthesis involve molecular oxygen and it was suggested that the beneficial effect of the hemoglobin on β-lactam production resulted from an increased level of intracellular oxygen.

Biosynthetic pathways have also been engineered to tailor β-lactams for the needs of chemical synthesis. 7-Amino cephalosporonic acid (7-ACA) is the starting material for a number of clinically important cephems, but a direct fermentation route to this compound has not been identified in nature. The current syntheses of 7-ACA from cephalosporin C relies on complex chemistry to cleave the α-aminoadipate side-chain without destruction of the β-lactam nucleus. To create a completely biological route to this compound, Isogai *et al.* (1991a,b) modified a cephalosporin-producing *A. chrysogenum* to express a D-amino acid oxidase from *Fusarium solini* and the cephalosporin acylase cloned from *Pseudomonas diminuti* (*Scheme 21*, top). Note that this metabolic pathway does not exist in any naturally-occurring organism. While the yield of 7-ACA from this engineered strain was very low (0.03 g/l), this strategy represents a very clever application of molecular biology to solving a chemical problem. A somewhat more efficient synthesis of 7-ACA was realized by Reeves and co-workers, who modified a penicillin-producing *P. chrysogenum* strain to express the expandase/hydroxylase and acetyl transferase enzymes from *A. chrysogenum* to create a hybrid pathway that did not exist in either of the starting organisms (*Scheme 21*, bottom) (Crawford *et al.*, 1995). In this approach, the host enzymes synthesized 6-aminopenicillanic acid by the normal route. This intermediate was transacylated with the acyl-CoA thioester derived from adipic acid that was included with the culture medium. The adipoyl-substituted penicillin was then converted by the *A. chrysogenum* expandase/hydroxylase complex to adipoyl-7-ADAC. Following acetylation of the side-chain hydroxyl group, adipoyl-7-ACA was isolated from the culture. The adipoyl side-chain was introduced because a commercially-available immobilized acylase was capable of removing this group to afford free 7-ACA. Unfortunately, a mass yield of 7-ACA was not given, although it was pointed out that 'application of classical strain improvement technology . . . has resulted in significant increases in the titers of the adipyl-cephalosporins, forecasting a commercial process' (Crawford *et al.,* 1995).

Another important class of cephalosporins features a non-hydroxylated methyl side-chain that is not directly available from large-scale fermentations. This would require an enzymatic ring expansion of the penicillin nucleus without subsequent hydroxylation (*Scheme 20*). Unfortunately, a number of attempts to disable only the hydroxylase activity of the bifunctional *A. chrysogenum* expandase/hydroxylase were unsuccessful (Cantwell *et al.,* 1992). For this reason, Queener and co-workers assembled a new metabolic pathway in *P. chrysogenum* by expressing an epimerase and a monofunctional expandase originally cloned from two *Streptomyces* species (*Streptomyces lipmanii* and *Streptomyces clavuligerus*, respectively) (*Scheme 22*, top)



Engineered Acremonium chrysogenum (Isogai et al., 1991):
Cephalosporin C
O2 + H2O
H2O2 + NH3
Fusarium solani
D-amino acid oxidase
H2O2
CO2 + H2O
Non-enzymatic
Pseudomonas diminuta
cephalosporin acylase
7-Amino
cephalosporanic acid
(7-ACA)



Scheme 21

(Cantwell *et al.*, 1992, 1995). The most efficient recombinant strain produced DAOC at a level of 2.5 g/l. A *Penicillium* host was chosen since the host strain synthesizes isopenicillin N but lacks an endogenous expandase pathway. One drawback to this approach was the difficulty in removing the α-aminoadipoyl side-chain from the final product. To avoid this problem, a different recombinant *Penicillium* strain was created by Reeves and co-workers using a similar strategy as that described above for the synthesis of 7-ACA. In this case, adipoyl-6-APA was converted by a monofunctional expandase from *S. clavuligerus* to adipoyl-7-ADCA (*Scheme 22*, bottom) (Crawford *et al.*, 1995). As before, the adipoyl side-chain could be removed from this fermentation product by an immobilized acylase to afford 7-ADCA.

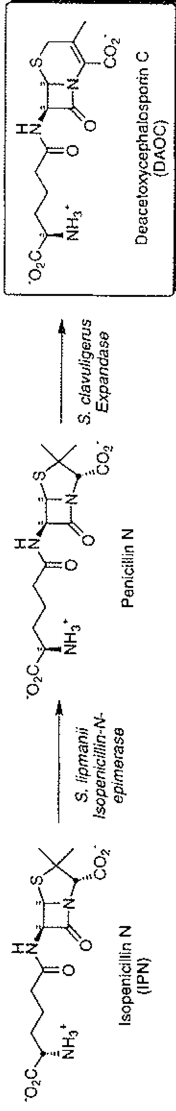
INDUSTRIAL CHEMICALS PRODUCED BY RECOMBINANT MICROBES

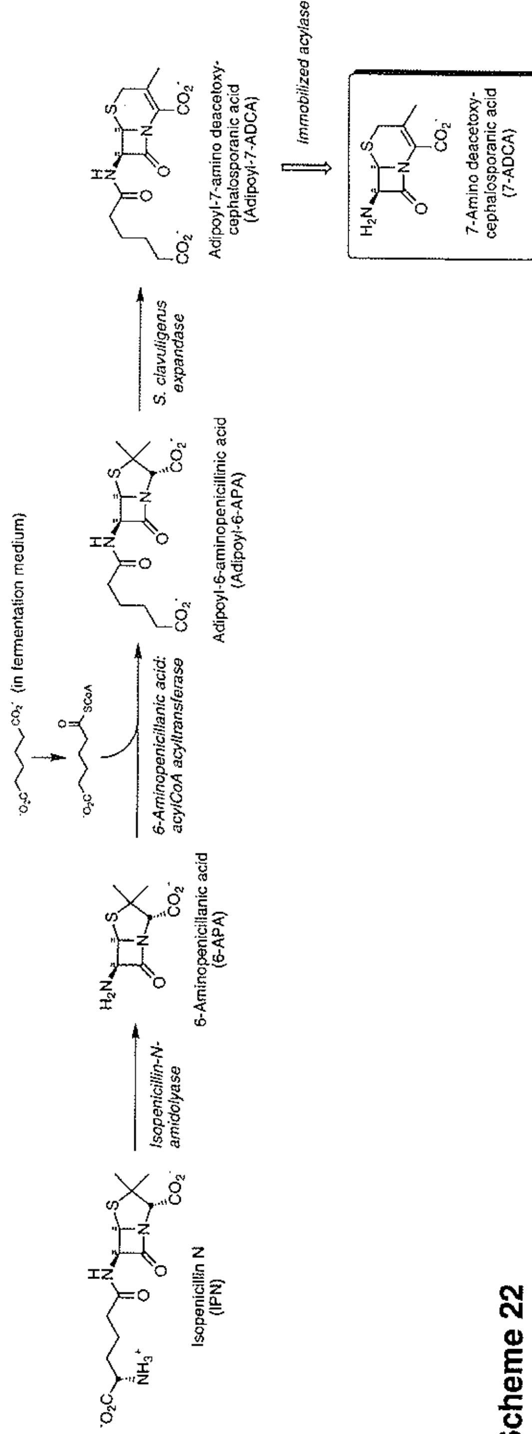
Fermentation routes to industrial chemicals have played important roles in the chemical industry, particularly in the early twentieth century. However, with the rise of the petroleum industry, many of these processes were abandoned in favor of less expensive routes based on petroleum feedstocks. More recently, a combination of political, economic and environmental concerns have renewed interest in non-petroleum routes to industrial chemicals. Since classical strain breeding alone has been unable to create organisms that synthesize these chemicals with costs competitive with existing technologies, recombinant DNA techniques have been applied to solving these problems. The scope of products available from this technology can be greatly extended by combining microbial fermentation with traditional organic synthesis. The overall result is the conversion of glucose into commodity chemicals. To make these routes successful, carbon flow must be maximized in both the existing as well as the newly-constructed biosynthetic pathways, which can be a difficult task.

Ethanol

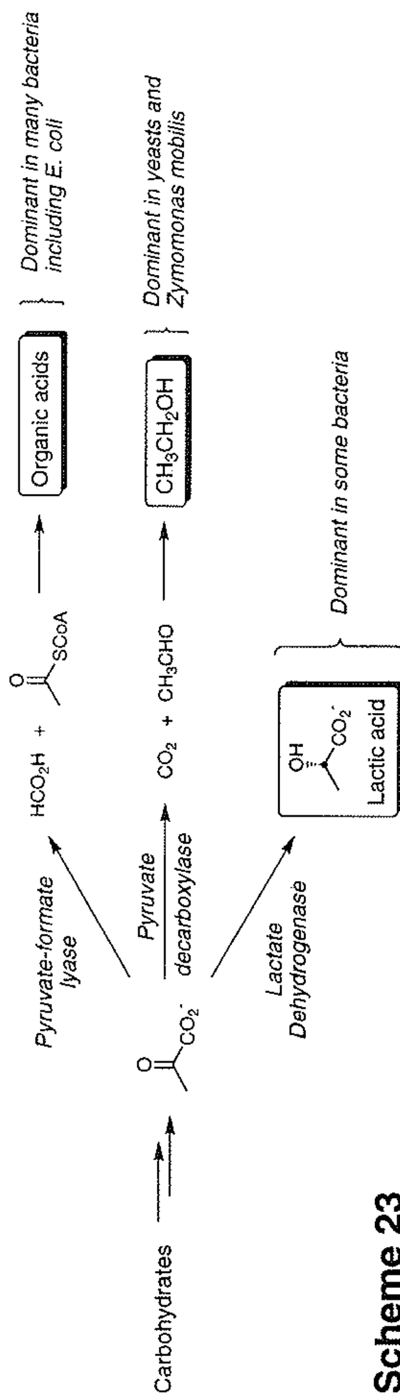
Sugar fermentation to ethanol in conjunction with bread, beer and winemaking is perhaps the oldest example of applied microbiology in human history. There has recently been an increasing interest in producing ethanol for industrial and fuel use by fermentation rather than from petrochemical feedstocks. In the production of high-volume, low-value chemicals, economic considerations are often more important that scientific ones. While yeast fermentation is an efficient route to ethanol, this organism is relatively limited in its carbon sources. On the other hand, a key attraction of metabolic engineering is the possibility of devising novel organisms that can convert 'waste' organic materials into ethanol that can be sold for a profit. Much of the research in this area has therefore been directed towards the use of alternate carbon sources as feedstocks, especially xylose and lactose. Xylose is the major carbohydrate component of hemicellulose, a plant-derived carbohydrate that is the second most abundant organic compound on Earth (after cellulose) and also one of the least valuable. As discussed above, lactose is the principle organic compound left in whey after protein and other useful products have been removed. Processes that can convert these two feedstocks into value-added products would therefore be extremely valuable.

Ethanol production is intimately connected with the growth of microorganisms





Scheme 22



Scheme 23

under oxygen-limited conditions. In this situation, cells contain excess reducing equivalents (primarily in the form of the reduced nicotinamide cofactor NADH). In order to continue carbon source assimilation, NADH must be converted into its oxidized form (NAD^+). Pyruvate, the end product of glycolysis, is the key metabolite (*Scheme 23*). In many bacteria, including *E. coli*, pyruvate is degraded into formate and acetyl-CoA by pyruvate-formate lyase. The latter is converted to organic acids such as acetate and succinate and these are excreted into the culture medium. In some bacteria, pyruvate is consumed by NADH-mediated reduction to lactate which is then excreted. Yeasts and a few bacteria such as *Zymomonas mobilis* use an alternate source for pyruvate consumption: decarboxylation to acetaldehyde which is then reduced by NADH to form ethanol. Most bacteria lack pyruvate decarboxylase and for this reason are unable to synthesize ethanol.

Two basic strategies have been explored for developing an economical process that converts lactose and/or xylose into ethanol. In the first, organisms that naturally grow on xylose and/or lactose are engineered to divert their carbon flow into ethanol. In the alternative approach, organisms that are already efficient ethanol producers but whose growth requires expensive sugars are engineered to utilize xylose and lactose. Because the primary emphasis of this review is on how recombinant microbes can solve chemical problems, the details of the recombinant DNA techniques and the biochemical characterization of the engineered microbes will not be discussed here; readers should consult the original papers for this information.

Pyruvate decarboxylase catalyzes the committed step in the formation of ethanol from pyruvate. Because the enzyme from *Z. mobilis* is highly efficient, this enzyme has been expressed in several bacterial hosts that naturally have the ability to grow on xylose but lack this enzyme, including *E. coli* (Bräu and Sahm, 1986; Neale *et al.*, 1988; Ohta *et al.*, 1991a; Alterthum and Ingram, 1989; Beall *et al.*, 1991), *Klebsiella planticola* (Feldmann *et al.*, 1989; Tolan and Finn, 1987) and *Klebsiella oxytoca* (Ohta *et al.*, 1991b) (*Table 7*). To increase ethanol production further, some workers have also expressed alcohol dehydrogenase II from *Z. mobilis* in the same cells. The latter enzyme reduces acetaldehyde to ethanol with high efficiency. With these improvements, production levels of ethanol have been improved to $\approx$ 50 g/l using xylose as the feedstock (*Table 7*). Importantly, the engineered *E. coli* strains can convert all of the sugars found in hemicellulose to ethanol (Alterthum and Ingram, 1989; Beale *et al.*, 1991). The most productive *E. coli* strain has also been tested for ethanol production using two industrially-relevant feedstocks, hardwood hemicellulose hydrolysate (Lawford and Rousseau, 1991) and cellulose (Padukone *et al.*, 1995). To make the process of hemicellulose fermentation even simpler, Ingram and coworkers have expressed a heat-stable xylanase gene in a *K. oxytoca* strain that also expressed *Z. mobilis* pyruvate decarboxylase and alcohol dehydrogenase II. Using a two-step fermentation, xylan was converted directly to ethanol without any chemical steps (Burchhardt and Ingram, 1992).

Another approach to converting waste biomass into ethanol by fermentation starts from organisms that efficiently convert expensive sugars to ethanol and introduces metabolic pathways for growth on inexpensive carbon sources. These studies have focused on *Z. mobilis* and various strains of baker's yeast (*S. cerevisiae*), since these organisms are among the most potent ethanol producers known (*Table 8*) (Porro *et al.*, 1992; Kötter *et al.*, 1990; Su *et al.*, 1989; Ramakrishnan and Hartley, 1993, Inlow *et*

Table 7. Ethanol production by bacteria expressing *Zymomonas mobilis* pyruvate decarboxylase

Strategy	Host	Sugar	Yield (g/l)	Reference
Expressed *Z. mobilis* pyruvate decarboxylase	*E. coli*	Glucose	2.3	Bräu and Sahm (1986)
Overexpressed *Z. mobilis* pyruvate decarboxylase	*K. planticola*	Xylose	17.8	Feldmann *et al.*, (1989)
Overexpressed pyruvate decarboxylase and alcohol dehydrogenase II from *Z. mobilis*	*E. coli*	Glucose	18	Neale *et al.*, (1988)
		Xylose	4	
Overexpressed *Z. mobilis* pyruvate decarboxylase	*K. planticola*	Xylose	25.1	Tolan and Finn (1987)
Overexpressed pyruvate decarboxylase and alcohol dehydrogenase II from *Z. mobilis*; strain breeding was used to increase protein expression	*E. coli*	Glucose	54	Ohta *et al.*, (1991)
		Xylose	42	
Overexpressed pyruvate decarboxylase and alcohol dehydrogenase II from *Z. mobilis*	*E. coli*	Glucose	34	Alterthum *et al.*, (1989)
		Xylose	56	
		Mannose	34	
		Galactose	32	
		Fructose	37	
		Arabinose	33	
Overexpressed pyruvate decarboxylase and alcohol dehydrogenase II from *Z. mobilis*	*K. oxytoca*	Glucose	48	Ohta *et al.*, (1991)
		Xylose	46	

Table 8. Ethanol-producing microbes with an expanded carbon source range

Strategy	Host	Sugar	Yield (g/l)	Reference
Expressed xylose reductase and xylitol dehydrogenase from *Pichia stipitis*	*S. cerevisiae*	Xylose	0.7	Kötter *et al.*, (1990)
Overexpressed *Xanthomonas albilineans* β-glucosidase	*Z. mobilis*	Cellobiose	6.1	Su *et al.*, (1989)
Overexpressed *E. coli* β-galactosidase	*S. cerevisiae*	Lactose	18.5	Porro *et al.*, (1992)
Overexpressed *Aspergillus niger* β-galactosidase	*S. cerevisiae*	Lactose	30	Ramakrishnan and Hartley (1993)
Overexpressed *Aspergillus awamori* glucoamylase	*S. cerevisiae*	Starch	45	Inlow *et al.*, (1988)
Overexpressed *Aspergillus awamori* glucoamylase; used distiller's yeast	*S. cerevisiae*	Starch	118	Cole *et al.*, (1988)
Overexpressed *Rhizopus oryzae* glucoamylase	*S. cerevisiae*	Starch	130	Ashikari *et al.*, (1989)

al., 1988; Cole *et al.*, 1988; Ashikari *et al.*, 1989). While attempts have been made to engineer *Z. mobilis* to produce ethanol from many carbon sources including lactose, raffinose, galactose, cellulose and xylose, these approaches have enjoyed only limited success (summarized by Yanase *et al.*, 1994) The sole exception was an engineered *Z. mobilis* strain engineered to produce ethanol from cellobiose (Su *et al.*, 1989). Most efforts have therefore focused on engineering baker's yeast. To produce ethanol from lactose, two groups have expressed a β-galactosidase in baker's yeast (Porro *et al.*, 1992; Ramakrishnan and Hartley, 1993). In the latter case, a strain of distiller's yeast was used as the host strain to provide better ethanol tolerance.

In a related vein, genetic engineering has also been used to improve the process of converting starch to ethanol. In the traditional approach, starch is gelatinized by cooking, liquefied by treating with α-amylase, saccharified to glucose by treatment with glucoamylase, then fermented to ethanol by *S. cerevisiae* (Cole *et al.*, 1988). However, by expressing glucoamylase in yeast, the saccharification step could be eliminated and ethanol was produced in high yields directly from soluble starch (Inlow *et al.*, 1988; Cole *et al.*, 1988; Ashikari *et al.*, 1989).

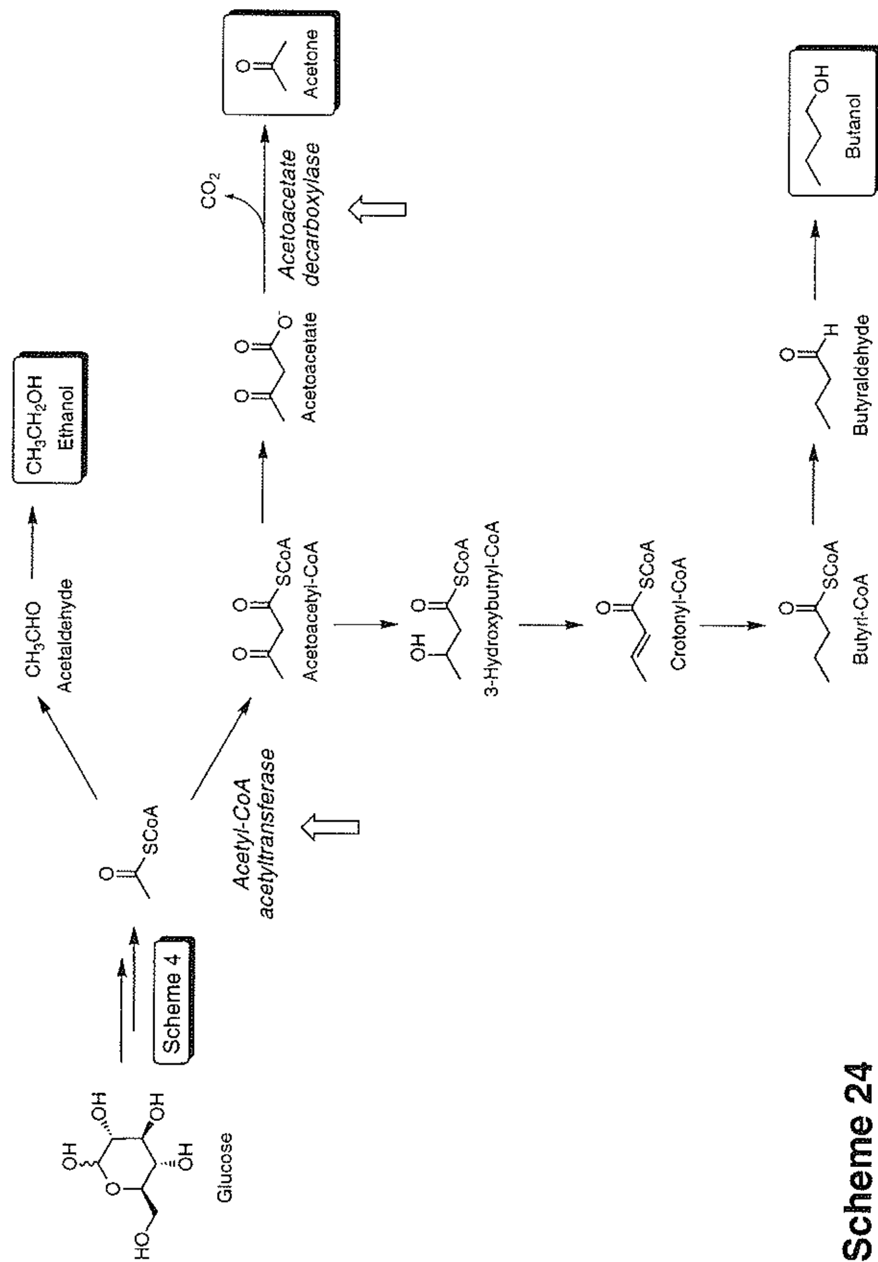
1,3-Propanediol

Cameron and co-workers Tong *et al.*, 1991) have reported the production of 1,3-propanediol by expressing two *Klebsiella pneumoniae* proteins in *E. coli*. While the level of product accumulated was relatively low (≤1.5 g/l), this could probably be improved by increased protein expression and improved fermentation methodology.

Acetone, butanol

Production of acetone, butanol and ethanol by fermentation using various *Clostridium* strains was an important process in the early part of this century to provide the raw materials for synthetic rubber. However, by 1960, this method had been completely supplanted by petroleum-based methods (Woods, 1995). The major disadvantages of the fermentation route are its low yields (typically 15 to 18 g/l total solvent), undesirable solvent ratios and relatively high substrate costs. While recombinant DNA technology would be an attractive way to address these problems, this is hampered by the lack of cloning technology available for *Clostridial* species. The biochemical pathways leading to ethanol, acetone and butanol are outlined in *Scheme 24* and are drawn to emphasize the kinship between this pathway and those of fatty acid and polyketide biosynthesis. Solvent production by *Clostridia* is their solution to regenerating NAD^+ from pyruvate under oxygen-limited growth conditions.

Based on the examples presented earlier, one obvious approach to increasing solvent production and simultaneously altering the ratios of the three solvents would be to increase the enzymes that catalyze the desired carbon flow. One key branchpoint is the condensation of two molecules of acetyl-CoA to acetoacetyl-CoA, catalyzed by acetyl-CoA acetyltransferase (*Scheme 24*). This step commits carbon flow to butanol and acetone at the expense of ethanol production. To increase the ratio of acetone produced by *Clostridium acetobutylicum*, Papoutsakis and co-workers (Mermelstein *et al.*, 1993) overexpressed acetyl-CoA acetyltransferase and acetoacetate decarboxylase. Under controlled fermentation conditions, a final acetone concentration of 8.7 g/l



Scheme 24

was attained by the recombinant strain; under similar conditions, the starting strain produced 4.5 g/l (Mermelstein *et al.*, 1993). If this level could be increased by an additional factor of three, the process would be competitive with petroleum-based routes (Woods, 1995).

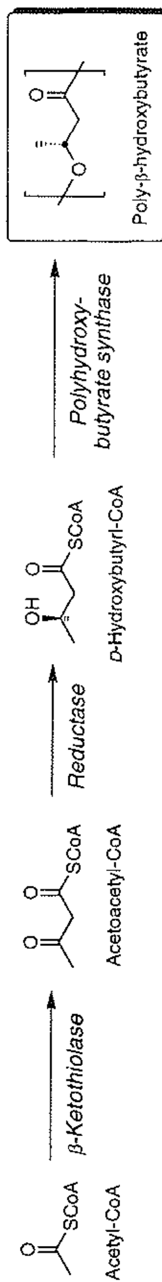
Poly-β-hydroxyalkanoates

A variety of bacteria produce poly-β-hydroxyalkanoates in response to limiting nutrients coupled with a large excess of carbon source. This pathway represents yet another way that microbes dispose of excess reducing equivalents in addition to those described above. However, in contrast to other microbes that excrete the reduced products (lactic acid, solvents, etc.) into the surrounding medium, these polymers are retained within the cells for later use as energy sources. Since their discovery in 1926, there has been a great deal of industrial interest in these polymers since they are completely biodegradable and can be made from renewable, non-petroleum feedstocks (Lee, 1996). Unfortunately, there are two major impediments to their widespread use: relatively inferior physical properties and high cost. The most common poly-β-hydroxyalkanoate is poly-3-hydroxybutyrate, produced on a commercial scale by the bacterium *Alcaligenes eutrophus*. A random co-polymer with 3-hydroxybutyrate and 3-hydroxyvalerate has much better flexibility, toughness and can be processed more easily (Byrom, 1987). These co-polymers can be made by including propionate in the culture medium. The challenge to biotechnology is to create more cost-effective routes to these polymers and to allow their rational tailoring to optimize their physical properties.

The biosynthetic pathway found in *A. eutrophus* involves three enzyme-catalyzed steps that are similar to those encountered in fatty acid and polyketide biosynthesis (*Scheme 25*). All three genes have been cloned from this organism and used to construct recombinant strains of *E. coli* (Schubert *et al.*, 1988; Slater *et al.*, 1988, 1992; Peoples and Sinskey, 1989; Lee *et al.*, 1994a, b; Zhang *et al.*, 1994), *K. oxytoca* (Zhang *et al.*, 1994), and *K. aerogenes* (Zhang *et al.*, 1994) that overproduce poly-β-hydroxyalkanoates. Very recently, a US patent (5,534,432) was issued for the production of these polymers in plants (Anon., 1996). The recombinant bacterial strains accumulate high levels of polymer (80% – 90% w/w of dried cell mass). To decrease the production cost further, strains were created that could produce polymers when grown on sucrose (Lee and Chang, 1993) or sugarcane molasses (Zhang *et al.*, 1994). To date, there have been no attempts to use genetic engineering to tailor the composition of these polymers and thereby improve their physical properties.

Benzoquinone and hydroquinone

To synthesize commodity aromatic compounds from glucose, intermediates in the aromatic amino acid biosynthetic pathway must be diverted into new products (Frost and Draths, 1995a, b). To channel overall carbon flow efficiently into the common pathway of aromatic biosynthesis, Draths and Frost engineered an *E. coli* strain to overexpress transketolase, DAHP synthase and 3-dehydroquinate synthase to remove restrictions on erythrose-4-phosphate, DAHP and 3-dehydroquinate production, respectively (Draths and Frost, 1990a, b). Since both hydroquinone and benzoquinone



Scheme 25

can be produced by chemical oxidation of quinate, the basic strategy was to divert the enhanced carbon flux of this pathway into this metabolite. To create a microbial pathway to quinate, quinate dehydrogenase from *K. pneumoniae* was expressed in the modified *E. coli* strain described above (*Scheme 26*, top) (Draths *et al.*, 1992b). To maximize carbon flux to quinate, the enzyme that competes for 3-dehydroquinate (3-dehydroshikimate synthase) was also disabled. Using this strain, quinate was produced at a level of 4.8 g/l. This intermediate was converted to both hydroquinone and benzoquinone by established chemical methods.

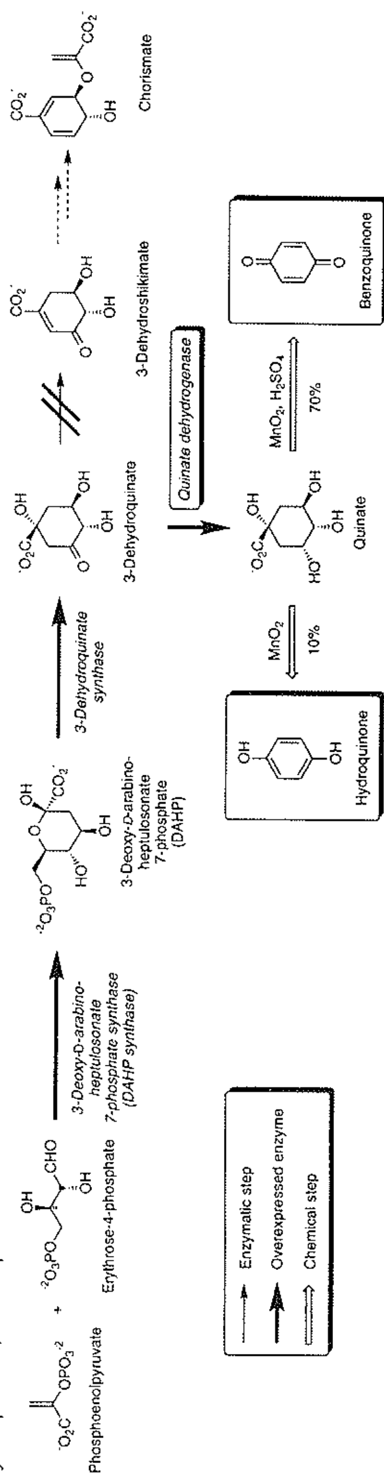
Catechol and adipic acid

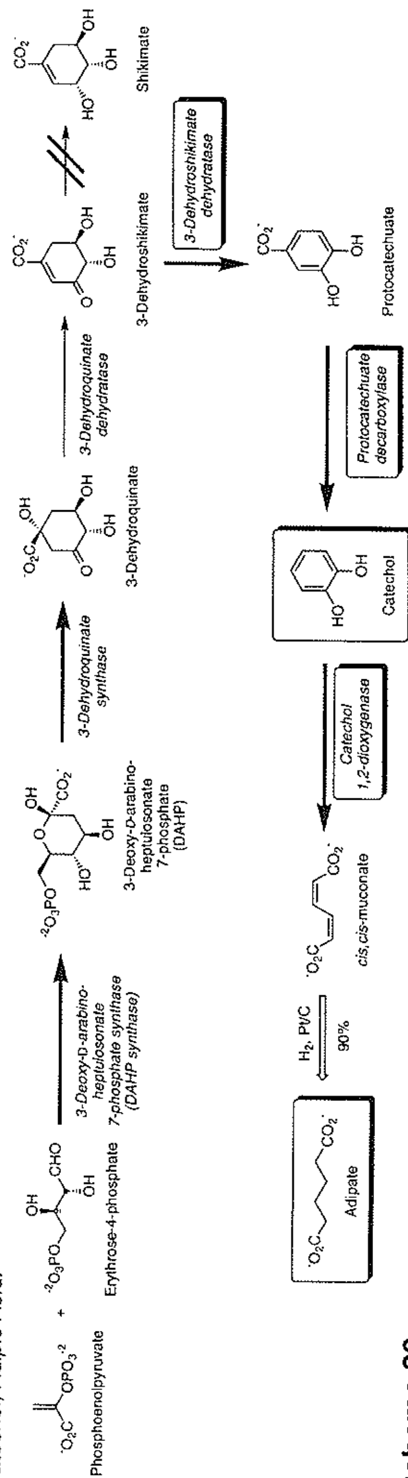
By combining two enzymes from a degradative pathway with a portion of the *E. coli* aromatic amino acid biosynthetic pathway, a fermentation route to catechol was created (*Scheme 26*, bottom) (Draths and Frost, 1991, 1995). Catechol was synthesized from 3-dehydroshikimate by aromatization followed by decarboxylation Since enzymes catalyzing these two steps are not made by *E. coli*, the appropriate *K. pneumoniae* genes were introduced by recombinant DNA technology. Furthermore, to avoid competing carbon flow into aromatic amino acids, shikimate dehydrogenase was inactivated. By using this engineered strain, catechol was produced at a level of 2.0 g/l. Unfortunately, this product was toxic to the cells, which complicated fermentations.

This hybrid pathway has also been extended to allow a simple fermentation/chemical route to adipate (*Scheme 26*, bottom) (Draths and Frost, 1995). *cis,cis*-Muconate, the end-product of fermentation, can be converted into adipate by simple catalytic hydrogenation. To convert catechol to *cis,cis*-muconate, catechol dioxygenase from *Acinetobacter calcoaceticus* was expressed in the *E. coli* strain that also expressed the two *Klebsiella* proteins. In small-scale experiments, *cis,cis*-muconate accumulated at a level of 2.4 g/l; this was subsequently reduced to adipate in 90% yield. While the cost of this fermentation route to adipate is well above that of the established chemical route (Draths and Frost, 1995b), the construction of this complex hybrid pathway demonstrates the power and the potential of metabolic engineering for large-scale chemical synthesis.

Engineered microbes as reagents in asymmetric organic synthesis

Most of the engineered microbes described to this point have been designed to convert carbohydrate feedstocks into useful chemicals. While this is an indisputably powerful approach, it is limited to compounds that can be derived from primary or secondary metabolism by a series of enzyme-catalyzed steps. Another important application of enzymes in organic chemistry is to catalyze reactions in which the substrates bear little or no resemblance to natural metabolites. In such cases, the enzyme substitutes for a 'chemical' reagent in an otherwise traditional synthetic route. In the past, this required the use of purified enzymes or whole microbial cells that express the enzyme of interest. As already noted (see Introduction), there are several disadvantages to both of these approaches. To overcome these deficiencies, we (Stewart *et al.*, 1996a,b) and others (Zylstra and Gibson, 1991; Herrmann *et al.*, 1994a,b; Hudlicky and Reed, 1995) have investigated the use of recombinant microbial cells as a way to allow





Scheme 26

enzymes to be easily used by practising organic chemists. In this approach, the recombinant microbe substitutes for an isolated enzyme and its associated cofactors. Since this is a relatively new strategy, some general aspects are described in the following section before specific examples are presented.

GENERAL CONSIDERATIONS IN DESIGNING USEFUL WHOLE-CELL SUBSTITUTES FOR ISOLATED ENZYMES

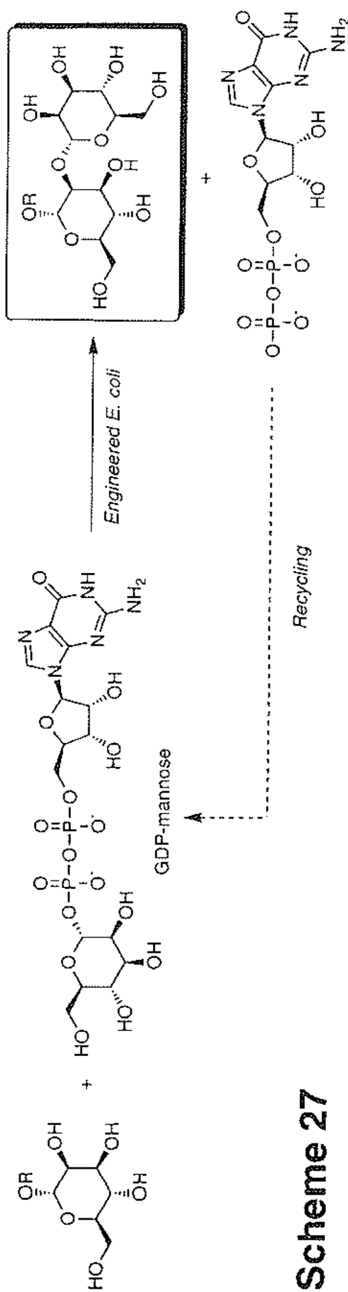
Before constructing an engineered microbe as a substitute for an isolated enzyme, it is important to weigh the time and effort required to create such a catalyst with its expected utility as a synthetic reagent. To aid in this process, we have developed a set of five general rules. *Rule #1: An engineered microbe will only be as useful as the enzyme it expresses.* One should therefore choose the target enzyme with care, with special emphasis on broad substrate specificity as well as high enantioselectivity. The idea is to create a microbe that accepts a broad class of substrates, not just one or two specific examples. *Rule #2: Inexpensive enzymes are poor choices.* This is self-explanatory; if a purified enzyme is readily available, there is little to be gained by creating a recombinant microbe as a substitute. Based on this rule, many common proteases and lipases are not suitable. *Rule #3: Membrane-bound enzymes are good choices.* Reconstituting membrane-bound proteins into artificial phospholipid membranes is often difficult and would be unfeasible for preparative-scale reactions. On the other hand, by using whole microbial cells, these proteins can be targeted to their correct locations automatically. *Rule #4: Enzymes that require cofactors are also good choices.* One of the strengths of whole-cell biotransformations is that cofactors are synthesized and regenerated by the cell. *Rule #5: Enzymes from 'difficult' sources are good choices.* Another strength of the recombinant microbe approach to using enzymes in synthesis is that the original source of an enzyme is irrelevant. This allows one to consider enzymes from virtually any source, so long as a small sample is available so that the appropriate gene can be isolated.

Both baker's yeast and *E. coli* have been used as hosts for expressing synthetically useful enzymes. The major advantages of yeast are chemists' familiarity with using natural varieties as synthetic reagents and its lack of reactivity toward most functional groups. On the other hand, *E. coli* offers better cloning technology and generally higher levels of protein expression. It may therefore be advantageous to express enzymes in both hosts. For small-scale experiments, yeast reagents are likely to be more convenient; conversely, for large-scale fermentations, *E. coli* may prove superior. Finally, other organisms may be better suited for specific enzymes. For example, chloroperoxidase uses hydrogen peroxide as one of its substrates. For synthetic purposes, this enzyme should be expressed in organisms that contain peroxisomes to ensure a sufficient supply of H_2O_2 for chemical catalysis.

EXAMPLES OF RECOMBINANT MICROBES AS SYNTHETIC REAGENTS

UDP-mannosyltransferase

The use of enzymes as catalysts for glycoside bond formation is already well-

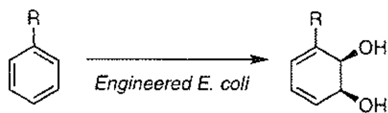


Scheme 27

established (Wong, 1995;Gijsen *et al.*, 1996). However, the limited number of commercially-available enzymes that catalyze useful glycosyl transfers remains an impediment to their wider use. Since a number of glycosyl transferase enzymes have been cloned, the possibility of using recombinant microbes to catalyze these reactions rather than the purified enzyme represents an attractive possibility. In the first example of this strategy, yeast α-1,2-mannosyltransferase was expressed.*E. coli* and the whole cells were used to synthesize a number of mannose-containing di- and trisaccharides (*Scheme 27*) (Herrmann *et al.*, 1994a,b). These recombinant cells could also be used to synthesize dimannosylated glycopeptides. One drawback to this approach is the high cost of the mannosyl donor (*ca.* $1,700/g). However, it should be possible to regenerate GDP-mannose *in situ* in the whole-cell mediated reactions using techniques developed earlier for reactions with the purified enzyme (Wang *et al.*, 1993).

Toluene dioxygenase

The oxidation of aromatics to*cis*-arene diols has already been discussed in the context of indigo production using *E. coli* cells engineered to express *Pseudomonas putida* napthalene dioxygenase (Ensley *et al.*, 1983). A similar dioxygenase system oxidizes toluene to the corresponding *cis*-diol (Gibson *et al.*, 1968). Whole cells of an *E. coli* strain that expresses all four proteins of the toluene dioxygenase system have been used to produce over 200 arene diol metabolites from aromatics added to the culture medium (Zylstra and Gibson, 1991). These chiral diols have been used extensively in organic synthesis and several reviews of this area have been published (Hudlicky and Reed, 1995; Hudlicky, 1996; Hudlicky and Thorpe, 1996). Furthermore, the fermentation process has been scaled up and several of the chiral arene diols are have become commercially available. This system provides one of the best examples of a successful marriage between chemistry and biology: apart from these dioxygenases, there is no way to produce enantiomerically pure arene diols in a single step from inexpensive starting materials.

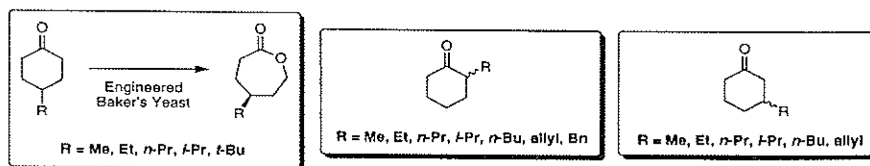


Scheme 28

Cyclohexanone monooxygenase

While several groups have recently reported small-molecule systems that catalyze asymmetric Baeyer-Villiger oxidations (Bolm and Schglingloff, 1995; Bolm *et al.*, 1994; Gusso *et al.*, 1994), enzyme catalysts for this reaction are more efficient and generally provide higher levels of enantioselectivity. Baeyer-Villiger enzymes from various bacterial species have been isolated and characterized (Königsberger *et al.*, 1990; Gagnon *et al.*, 1994; Grogan *et al.*, Donoghue *et al.*, 1976; Adger *et al.*, 1995). The enzyme from *Acinetobacter sp.* NCIB 9871 (Donoghue *et al.*, 1976) has been studied most thoroughly and is considered the prototype for this class of monooxygenases (Schwab, 1981; Ryerson*et al.*, 1982; Schwab*et al.*, 1983, Branchaud

and Walsh, 1985; Latham and Walsh, 1987). While several groups have reported applications of this enzyme to organic synthesis (summarized by Stewart *et al.*, 1996a), the need to isolate the enzyme and its requirement for NADPH make preparative-scale reactions difficult. The use of whole cells of the original microorganism is complicated by its pathogenicity, the possibility of overmetabolism of the lactones and the need to grow the organism on cyclohexanol (which complicates product isolation). To avoid all of these problems, and to create a simple reagent that could be used by chemists with no training in microbiology, this enzyme was expressed in baker's yeast (Stewart *et al.*, 1996a). Whole cells of the designer yeast were used to convert several 4-substituted cyclohexanones to the corresponding (*S*)-lactones in high optical purities and good yields (*Scheme 29*) (Stewart *et al.*, 1996a). More recently, the same strain was used to effect very efficient kinetic the resolutions of several racemic 2-substituted cyclohexanones (Stewart *et al.*, 1996b). Surprisingly, with one exception (Schwab *et al.*, 1983), the enzymatic Baeyer-Villiger oxidation of this series of compounds had not been previously explored. The same yeast has also been applied to the asymmetric Baeyer-Villiger oxidations of a series of 3-alkyl-substituted cyclohexanones. An enzymatic system is especially useful for this class of compounds where regioselectivity is an issue (C.A. Martinez, M.M. Kayser and J.D. Stewart, unpublished results).



Scheme 29

Concluding remarks

This review has been designed to provide a broad overview of the field of metabolic engineering and to show how various problems in organic chemistry have been solved by a biocatalytic approach. While this broad coverage has meant that many details had to be omitted, it is hoped that the reader will have gained an understanding of how engineered microbes fit into the larger picture of modern organic chemistry. In some cases, such as the large-scale synthesis of enantiomerically pure amino acids, their superiority is obvious. However, for other products, such as acetone or engineered polyketide antibiotics, the choice is not quite so clear, and both chemical and biological approaches have merits and demerits that must be weighed. However, with advances in cloning technology, it is expected that biological catalysts will find increasing use in large-scale chemical synthesis.

The examples cited above have also pointed out a number of areas that require further research to make engineered microbes more useful as chemical catalysts. (1) *More efficient conversion of carbohydrates to products of secondary metabolism.* Efforts to engineer efficient production of primary metabolites, especially amino acids, have been very successful. On the other hand, efforts to engineer the production of secondary metabolites such as antibiotics or 2-keto-L-gulonate (the precursor of

ascorbic acid) have not addressed the issue of increased precursor supply from primary metabolism as a means of increasing production levels. The importance of this issue is apparent from the success of Frost (in increasing production in engineered metabolic pathways by focusing on precursor supply (Snell *et al.*, 1966). (2) *Better cloning technology for Clostridial species.* Solvent production by *Clostridia* could be made competitive with petroleum-based routes by relatively small increases in productivity. The efficient channeling of carbon flux into solvent production by this organism suggests that it may be a useful host for other engineered metabolic pathways that lead from acetyl-CoA. At the moment, however, the lack of tools for recombinant DNA approaches to this organism is a serious impediment. (3) *Production of secondary metabolites in Corynebacteria.* The successful use of these species in amino acid production suggests that they may also be useful for expressing products of secondary metabolism. Research in this area would be aided by existing strains that efficiently channel carbon flux into specific pathways of primary metabolism. (4) *Production of biosurfactants in conjunction with synthetically useful enzymes.* The insolubility of most organic compounds in water complicates many biocatalytic transformations and exogenous reagents are often added, which increases process costs. On the other hand, bacteria that grow on hydrophobic carbon sources such as crude oil secrete surfactants that emulsify and solubilize these compounds. By introducing the metabolic pathways for biosurfactant production into strains that also catalyze useful chemistry, solubility problems could be economically overcome. While this is not yet possible, the cloning and expression of these genes is currently an area of active research (Fletcher, 1992; Sarney and Vulfson, 1995).

Acknowledgments

The National Science Foundation (CHE-9513349) and the University of Florida are gratefully acknowledged for supporting the author's research in this area. The development of baker's yeast as a general catalyst for asymmetric synthesis has been a collaborative effort with Prof. Margaret Kayser (University of New Brunswick), and her helpful comments on this manuscript are appreciated.

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