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REVIEW PAPER

Shu-Jen Chiang

Strain improvement for fermentation and biocatalysis processes by genetic engineering technology

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Abstract Twenty years ago, the first complete gene cluster encoding the actinorhodin biosynthetic pathway was cloned and characterized. Subsequently, the gene clusters encoding the biosynthetic pathways for many antibiotics were isolated. In the past decade, breakthroughs in technology brought that generation of rationally designed or new hybrid metabolites to fruition. Now, the development of high-throughput DNA sequencing and DNA microarray techniques enables researchers to identify the regulatory mechanisms for the overproduction of secondary metabolites and to monitor gene expression during the fermentation cycle, accelerating the rational application of metabolic pathway engineering. How are the new tools of biotechnology currently being applied to improve the production of secondary metabolites? Where will this progress lead us tomorrow? The use of whole cells or partially purified enzymes as catalysts has been increased significantly for chemical synthesis in pharmaceutical and fine-chemical industries. The development of PCR technologies for protein engineering and DNA shuffling is leading to the generation of new enzymes with increased stability to a wide range of pHs, temperatures and solvents and with increased substrate specificity, reaction rate and enantioselectivity. Where will this emerging technology lead us in the twenty-first century?

Keywords Biocatalysis · Enzyme engineering · Fermentation · Pathway engineering · Secondary metabolites

S.-J. Chiang
Fermentation and Biocatalysis Development,
Technical Operations, Bristol-Myers Squibb Company,
P.O. Box 4755, Syracuse, NY 132210-4755, USA
E-mail: shu-jen.chiang@bms.com
Tel.: +1-315-4329575
Fax: +1-315-4324891

Metabolic pathway engineering for the production of secondary metabolites and chemicals

As we commemorate the 50th anniversary of the discovery of the double-helical structure of DNA, it is also worthwhile to note that, about 30 years ago, the pioneering work of Berg, Boyer and Cohen led to the development of recombinant DNA technology [1, 2]. Since that time, genetic engineering has been greatly advanced and widely applied in many industrial microbiological interests. When the production of hybrid antibiotics by genetic engineering was first reported by the collaboration of Hopwood et al. [3], there was a great expectation that combining genes from different antibiotic pathways would generate many novel antibiotics. After 19 years, many novel antibiotics were indeed generated and detected on the laboratory scale, nevertheless their low yield prohibited their production on the industrial scale. In this review article, I would like to discuss several examples in the areas of strain improvement and metabolic pathway engineering that have been successfully used for industrial-scale production.

Strain improvement

Manipulation of transcriptional regulators Similar to the gene expression control of operons for carbohydrate metabolic pathways, the regulatory proteins for the expression of secondary metabolites can be categorized into two classes, positive and negative control [4]. In a liquid-grown culture of *Streptomyces coelicolor*, the presence of pathway-specific regulatory genes, *redD* and *actII*-ORF4 [5, 6], are required for the production of the pigmented antibiotics undecylprodigiosin and actinorhodin, respectively, demonstrating examples of positive control. In contrast, negative control requires the removal of repressor proteins in order to activate the genes encoding secondary metabolite biosynthesis [7]. Classic UV or chemical mutagenetic programs for strain improvement may select for mutants with an elevated

production of positive regulatory proteins or the inactivation of repressor proteins. There are no examples of modifying regulatory genes in industrial production strains by a genetic engineering approach.

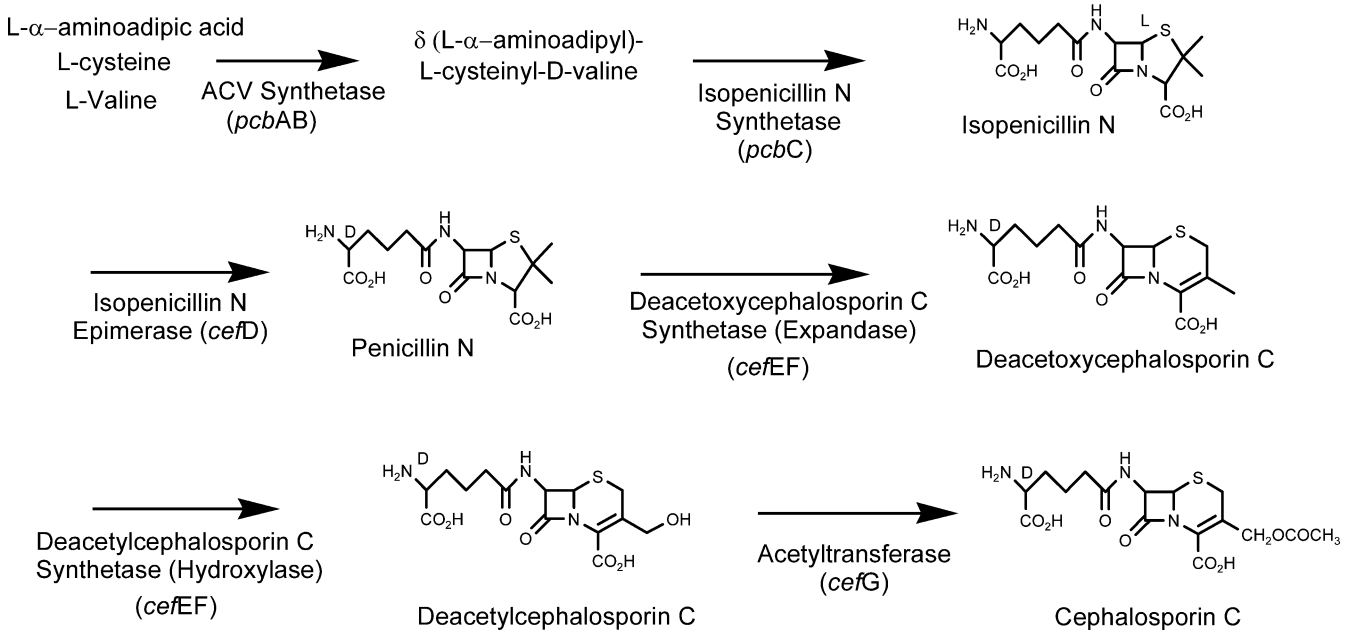
Increase gene dosage of rate-limiting enzymes Since the discovery of cephalosporin C in 1945, semi-synthetic cephalosporins produced by the acylation of the nucleus of cephalosporin C, 7-amino-cephalospranic acid (7-ACA), have become one of the most important classes of antibiotics due to their low toxicity and effectiveness against bacterial infection. The cephalosporin C biosynthetic pathway in *Acremonium chrysogenum* is well characterized both biochemically and genetically (Fig. 1). Deacetoxycephalosporin C (DAOC) is formed as an intermediate in cephalosporin C biosynthesis. In this pathway, penicillin N is converted to DAOC and then to deacetylcephalosporin C (DAC) by the bifunctional expandase/hydroxylase enzyme. During cephalosporin C fermentation, DAOC accumulates in the fermentation broth to a concentration of 1–2% of the cephalosporin C produced. When the cephalosporin C is extracted from the fermentation broth and hydrolyzed to 7-ACA, the DAOC present is converted to 7-amino-deacetoxy-cephalosporanic acid (7-ADCA), an undesirable contaminant of 7-ACA. By genetically engineering strains with two copies of the expandase/hydroxylase gene, Basch and Chiang [8] were able to double the amount of expandase/hydroxylase transcript and reduce the level of DAOC present in the fermentation broth to 50% of the control in large production fermentors. The recovery and purification of these broths and subsequent chemical conversion to 7-ACA resulted in a significant reduction in 7-ADCA contamination. The increase in expandase/hydroxylase enzymes reduced the accumulation of DAOC intermediate. However, it had no impact on the productivity of cephalosporin C, suggesting that the expandase/hydroxylase enzyme step may not be rate-limiting for cephalosporin C production [9, 10].

Gene mutation to enhance the yield of the desired product Avermectin and its analogues are produced by *S. avermitilis* and are major commercial products for parasite control. The avermectin analogue doramectin (B1), which is sold by Pfizer as Dectomax, is co-produced with an undesired analogue (B2) during fermentation, at a B1:B2 ratio of 0.6 (Fig. 2). The B1 molecule has a double bond between C-22 and C-23 and the B2 molecule contains a saturated C–C bond and a hydroxyl group at C-23. Although the function of the *aveC*gene is dehydration at C-22 and C-23, the mechanism for determining the B1:B2 ratio remains unclear [11]. Using an error-prone polymerase chain reaction (PCR) to introduce random mutations into the *aveC* gene and transform the mutated genes into an *aveC* deletion strain, Stutzman-Engwall et al. [12] demonstrated that one of the *aveC* mutants yielded a B1:B2 ratio of 2.5, which is a four-fold improvement over the wild-type *aveC* strain. At the 2003 SIM annual meeting, del Cardayre (personal communication) reported that subsequent manipulation of the *aveC* mutants, using gene shuffling, resulted in a 26-fold increase in the B1:B2 ratio over the wild-type *aveC* strain.

Modification of natural metabolites

A number of semi-synthetic cephalosporins, such as cephalexin, cefadroxil and cephradine, are made from 7-ADCA, in turn made by a chemical ring expansion of

Fig. 1 Cephalosporin C biosynthetic pathway



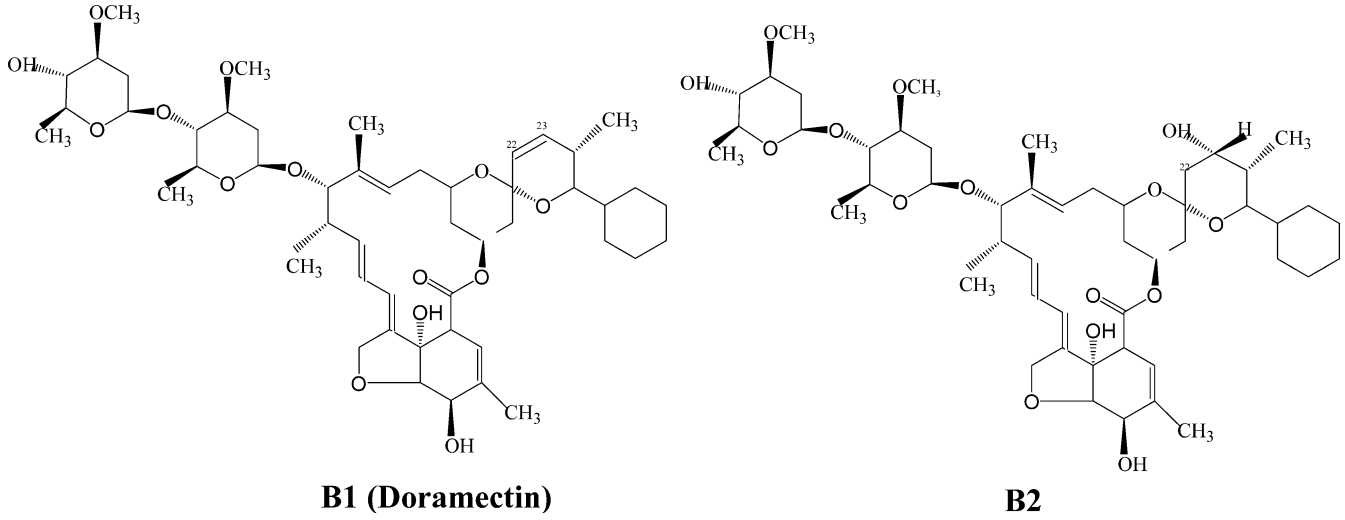


Fig. 2 Avermectins

6-aminopenicillanic acid (6-APA). In 1990, Cantwell et al. [13] proposed a process of ring expansion of penicillin G or V to DAOC, using a modified expandase enzyme, followed by enzymatic hydrolysis to give 7-ADCA. Later, a collaboration of Merck and Panlabs showed that a *Penicillium chrysogenum* strain transformed with a *S. clavuligerus* expandase gene could produce adipyl-7-ADCA in the presence of adipic acid [14]. Since the substrate recognition of the *Streptomyces* expandase enzyme is limited to the analogues of penicillin N, the expandase transformants could not expand penicillin G. Adipic acid was added to the fermentation broth to produce adipyl-6-APA that was converted to adipyl-7-ADCA by the expandase enzyme (Fig. 3). The adipyl side-chain of this product could then be cleaved with amidase to produce 7-ADCA.

Sutherland et al. [15] at DSM further demonstrated that a mutated *Streptomyces* expandase gene, modified by enzyme engineering, is able to convert penicillin G to phenylacetyl-7-ADCA, which could then be hydrolyzed to 7-ADCA, by penicillin G amidase (Fig. 3). DSM is building a new plant at its Delft complex for the

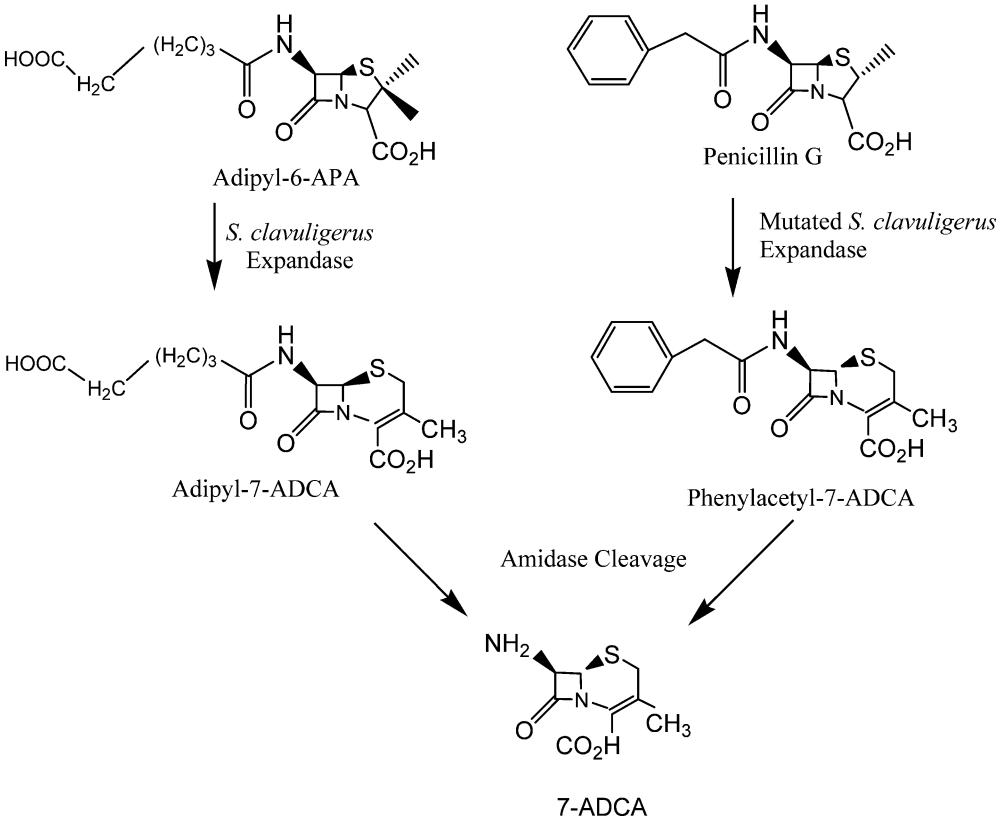


Fig. 3 Recombinant *P. chrysogenum* strain for the fermentation of 7-ADCA precursors

fermentation of phenylacetyl-7ADCA and its enzymatic cleavage, to produce 7-ADCA at a substantially lower cost.

Expression in a heterologous host

Whole or partial pathway expression in a heterologous host The gene clusters encoding biosynthetic pathways for many antibiotics have been isolated. In most cases, the production microorganism was not amenable to efficient genetic manipulation. Therefore, the development of heterologous expression in commonly used industrial strains for productivity improvement is highly desirable. In order to achieve this objective, the following factors should be considered in the choice of heterologous expression systems. The first is the efficient expression and correct protein folding/assembly of the enzyme in the new host. Unless the promoter sequences of the expressed genes can be replaced with the new host promoters, a closely related host should be used for the proper promoter recognition by the host RNA polymerase. Second, the precursors for the biosynthesis of the desired product must exist in the new host.

Significant advancement has been made in the heterologous expression of polyketides. A genetically engineered host of *S. coelicolor*, CH999, has been constructed by deleting the entire actinorhodin gene cluster and thereby eliminating background actinorhodin synthesis. The entire or partial biosynthetic gene clusters of frenolicin, tetracenomycin, oxytetracycline, R1128 (a non-steroid estrogen receptor antagonist), erythromycin, picromycin/methymycin and oleandomycin have been expressed in the *S. coelicolor* CH999 host. However, productivity to date is too low for commercial production [16].

Artificial pathway engineering 1,3-Propandiol is a monomer that can be used for the production of polyester fibers, polyurethanes and cyclic compounds. The biological production of 1,3-propanediol from glycerol has been well studied in *Klebsiella pneumoniae* and *Clostridium butyricum*. Since glycerol is an expensive starting material, a two-stage fermentation process was developed using *Saccharomyces cerevisiae* first to produce glycerol and *K. pneumoniae* for the subsequent conversion to 1,3-propanediol [17]. *S. cerevisiae* produced glycerol from dihydroxyacetone 3-phosphate using two enzymes: dihydroxyacetone 3-phosphate dehydrogenase and glycerol 3-phosphate phosphatase. The production of 1,3-propanediol from glycerol in *K. pneumoniae* requires glycerol dehydratase and 1,3-propanediol dehydrogenase. However this two-stage fermentation process for the production of 1,3-propanediol is still not cost-competitive with the chemical process.

In a collaboration of DuPont and Genecor, a recombinant *Escherichia coli* strain was constructed with an artificial operon, consisting of the genes encoding for these four enzymes, to produce 1,3-propanediol in a single fermentation step (Fig. 4) [17, 18]. A very effective and cheap fermentation process was developed; and DuPont is in the process of building a new fermentation

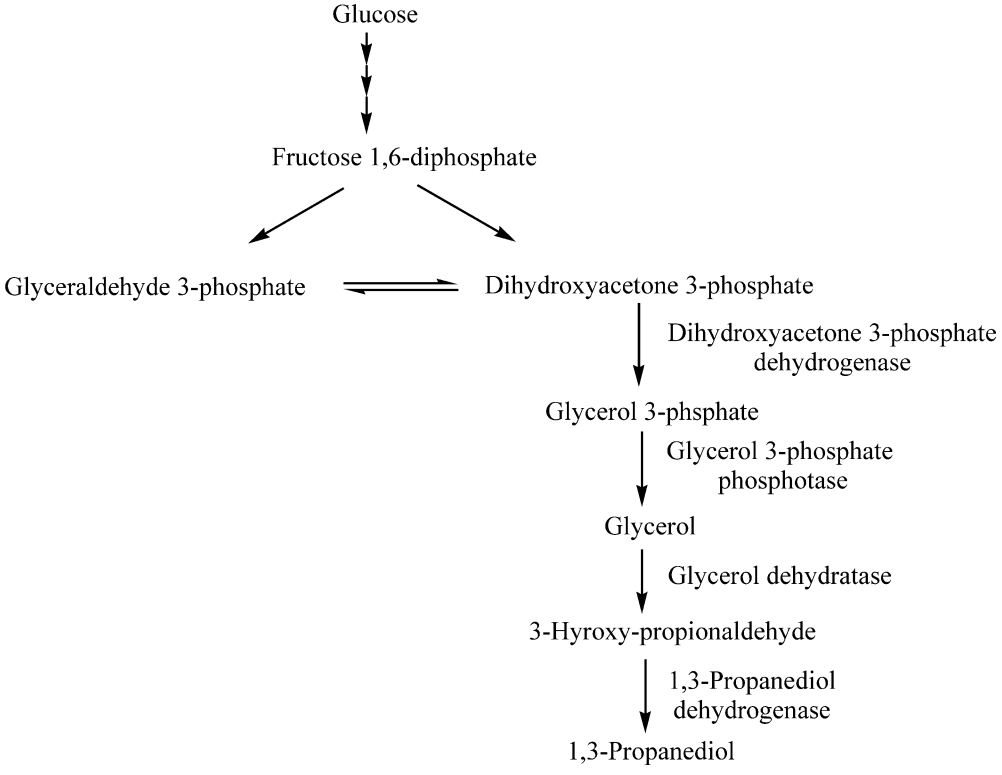
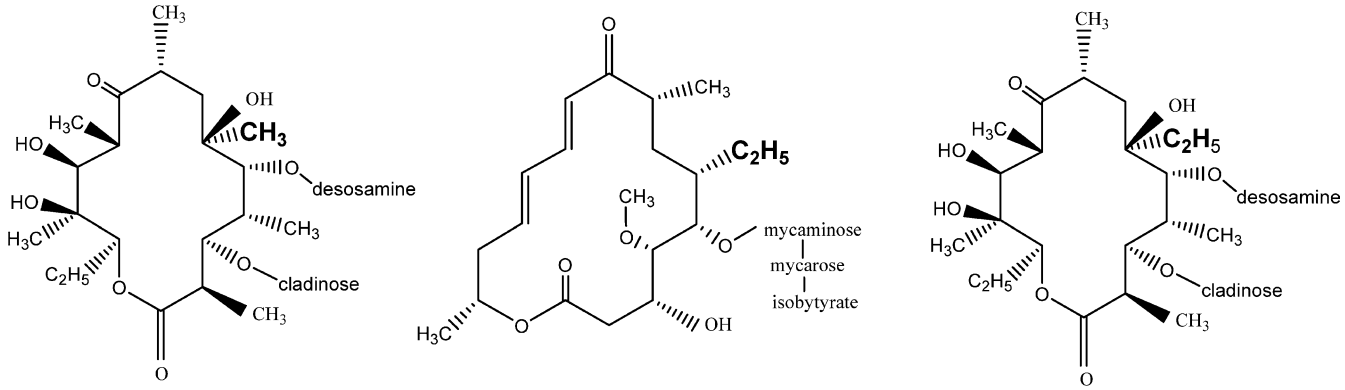


Fig. 4 Recombinant *E. coli* strain for the direct fermentation of 1,3-propanediol



Erythromycin A Niddamycin 6-Ethylerythromycin A

Fig. 5 Erythromycin and ethyl-substituted erythromycin derivatives

plant in Blair, Neb., for the production of 1,3-propanediol, which is a key ingredient in DuPont's new polymer, Sorona.

Domain modification for making hybrid metabolites

The successful cloning of the type I polyketide synthase (PKS) genes of several macrolide antibiotics revealed that PKS enzyme complexes usually comprise several large multifunctional polypeptides, each of which contains repeated modules. Each module contains the enzymatic activities necessary for one condensation and, in some cases, subsequent reduction of the extender to the growing polyketide chain [19]. Hybrid PKSs have been constructed through the replacement of acyltransferase (AT) domains with those that specify different starter or extender units. These manipulations result in the production of novel polyketides. For example, Katz and associates demonstrated the replacement of the methyl side chain at the C-6 position of erythromycin with an ethyl moiety to produce 6-ethylerythromycin A (Fig. 5) [20]. The methylmalonate-specific AT domain of the erythromycin PKS was replaced with an ethylmalonate-specific AT domain from the nidamycin PKS. When the recombinant culture was supplied with precursors of ethylmalonate, a small quantity of 6-ethylerythromycin A was produced with erythromycin A. Subsequently, the introduction of a crotyl-CoA reductase gene for making the precursors of ethylmalonyl-CoA led to the sole production of 6-ethylerythromycin A, however, the yield is still too low for commercial production.

In the attempt to make hybrid polyketide pathways, even after the recombinant strain is engineered to produce an ample supply of the required precursors, the yield of hybrid polyketide is still very low. After cyclization of the polyketide linear chain to aglycon, the enzymes carrying the subsequent aglycon modification or side-chain addition may not be able to recognize the new polyketide aglycon structure and enzyme engineering of these modification enzymes is necessary to boost the productivity of the novel compounds (see the next section for enzyme engineering).

Conclusion

In this review article, I have only listed a few successful examples and it is not my intention to provide an extensive review covering all successful experiments. In the above examples, it is worthwhile to note that a simple increase in biosynthetic gene copies could never enhance productivity in a well developed industrial strain. However, productivity increases were observed in wild-type strains or strains in the early development stages [21]. Many years of classic mutagenesis have selected improved strains with increased precursor pools, better metabolic flux and other host mutations to allow a higher productivity of a metabolite.

Due to the increase in drug-resistant bacteria, most antibiotics cannot be used in their natural form and require chemical derivation to make them effective anti-infective agents. By introducing genes encoding enzymes to modify these natural metabolites, many recombinant strains have been constructed, which only require one-step fermentation to make the product. This area will be one of the most fruitful applications of future strain improvement by genetic engineering.

Most cases of secondary metabolite biosynthesis are under very tight regulation. Unless the flux of pathway biosynthesis is continuous, the accumulation of any intermediates usually causes feedback inhibition of the synthesis of that intermediate or breakdown of that intermediate. For example, DAC is far more stable in fermentation broth than cephalosporin C [22]. However, blocking the last step of cephalosporin C biosynthesis for the production of DAC causes the oxidation of DAC by the bifunctional expandase/hydroxylase enzyme (Fig. 1) [23]. An alternative approach to produce DAC is the addition of an acetyl esterase enzyme from *Rhodosporidium toruloides* to the fermentation, resulting in the accumulation of DAC and an increase in antibiotic yield of ~40% [22].

The genetic stability of the transforming plasmid in the transformants is critical to ensure the production of recombinant products during fermentation. Autonomous replicating plasmids were commonly used for the construction of recombinant strains. These plasmids usually carry genes conferring resistance to antibiotics for the selection of transformants; and the continuous presence of these antibiotics is required during fermentation to maintain the transforming plasmids. The removal of these antibiotics in the purification of the final product increases the cost of the process and often results in United States Food and Drug Administration regulatory issues. It has been established that the transforming plasmid can be forced to integrate into the host chromosome and remain genetically stable, even in the absence of selective antibiotics. This approach eliminates the concern of antibiotic addition and should always be considered for the construction of recombinant strains.

Enzyme engineering for biocatalysis

The use of enzymes in pharmaceutical industries

Microbial cell-mediated fermentation and biodegradation have been used in the food, tanning, textile and wine industries for many centuries. In the twentieth century, biochemical investigations revealed the enzymes that are the biocatalysts for these biochemical reactions. Using isolated enzymes to catalyze a single-step reaction in vitro is feasible, although the high cost, complexity and slow reaction time of many enzymes make them less competitive with existing chemical processes in pharmaceutical or fine chemical industries. Recombinant DNA technology enables the production of enzymes at levels 100-fold greater than the native expression, making them available at low cost and in large quantities.

Table 1 The use of enzymes in pharmaceutical industries

Enzyme	Pharmaceutical use
Nitrile hydratase	Nicotinamide
Dehydrogenase	Omapatrilat, tert-leucine, Trusopt (dorzolamide)
Amidase, acylase	6-APA, Cefzil (cefprozil), Duricef (cefadroxil)
Lipase, esterase	Paxil (paroxetine), Captopril, posaconazole
Nitrilase	Ritulin (methylphenidate)
Protease	Ziagen (abacavir)
Dehalogenase	Lipitor (atorvastatin)
P450 monooxygenase (whole cells)	Pravacol (pravastatin), steroids, atenolol
Adenosine deaminase	Videx (didanosine)
Trypsin	Humulin (human insulin)
D-Hydantoinase	D-Phenylglycine, D-amino acids
D-Amino acid oxidase	Keto acids

Table 1 illustrates some examples of enzymes that have been used in pharmaceutical industries.

Application of biocatalysis

Enzyme-mediated reactions can be performed in three different forms.

Isolated enzymes Soluble enzymes can be partially purified from cells and used in a reaction mixture as free enzyme or immobilized on a solid carrier, which allows easy recovery for repeated usage.

Whole cells Intact cells are removed from the growth medium but still maintain most metabolic activities. This is most useful for reactions requiring electron transport, regeneration of cofactors, multi-enzyme steps, or the use of enzymes not amendable to isolation (e.g., multimeric or membrane-bound).

Active fermentation Actively growing cells are sometimes used for enzyme reactions. This system can only be employed if the components in fermentation medium do not interfere with the enzyme reaction. The addition of substrate to fermentation broth is the most direct means of biocatalysis.

Cloning and expression in commonly used industrial hosts

Microorganisms isolated from diverse environments represent a source of enzymes that can be used for industrial process chemistry. Using high-throughput screening (HTS) methods, new biocatalysts can be found from these microorganisms. But many microorganisms are not easily cultivated in laboratory conditions or their enzyme yield is too low for economical use. Using recombinant DNA technology, cloning the genes encoding these enzymes and heterologously expressing them in commonly used industrial strains have become a common practice.

The enzyme penicillin V amidase (PVA) is used for the hydrolysis of penicillin V to 6-APA (Fig. 6). The secreted form of the PVA enzyme was isolated from a soil isolate, *Fusarium oxysporum*strain XF4. Production of the PVA enzyme can be induced 50-fold by the addition of phenoxyacetate; and this induction takes place at the transcriptional level. Both genomic and cDNA clones of the PVA gene were isolated. A recombinant plasmid was constructed with two copies of the PVA gene and the phleomycin-resistance gene for selection of fungal transformants. A heterologous host, *F. oxysporum* f.sp. *lycopersici* (ATCC 16322), was used for DNA transformation. Chiang et al. [24] demonstrated that, with a significant increase of the amount of phenoxyacetate, the fermentation of

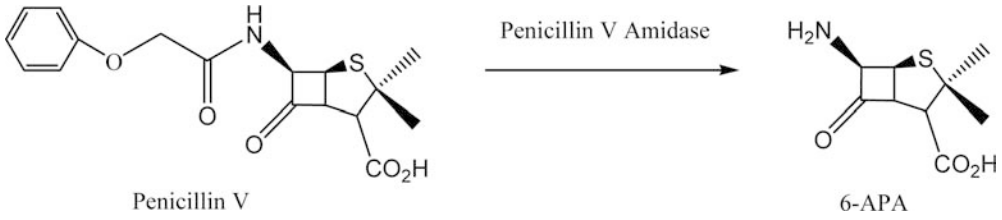


Fig. 6 Amidase-mediated enzymatic hydrolysis of penicillin V to 6-APA

phleomycin-resistant transformant in large-scale fermentors produced a nine-fold increase in PVA over the natural host.

Objectives of enzyme engineering

Although the use of recombinant DNA technology significantly lowers the cost of enzyme production, the applications of enzymes are still limited. Most chemicals with industrial interest are not natural substrates for these enzymes. If a desired enzyme activity is found, the yield is often low. Moreover, enzymes are not usually stable in harsh reaction conditions, such as pH higher or lower than physiological pH 7, high temperature or the presence of organic solvents required to solubilize many substrates. With recent advances in PCR technology, site-specific and random mutagenesis are readily available to improve enzyme stability in a wider range of pH and temperature and tolerance to a variety of organic solvents. Since a large quantity of enzyme can be obtained by recombinant expression, X-ray crystallography can facilitate the understanding of the tertiary structure of an enzyme and its substrate binding/recognition sites. This information may assist a rational design of the enzyme, predicting amino acid changes for altering substrate specificity, catalytic rate and enantioselectivity (in the case of chiral compound synthesis). Two PCR-mediated methods, random or saturated site-specific mutagenesis and gene shuffling, are generally used for generating mutants. This process is called molecular breeding or directed evolution.

Methods used for enzyme engineering

Saturated site-specific or random mutagenesis Single or multiple mutations can be introduced into a gene using oligonucleotide primers with altered base pairs at one or several specific sites. Using error-prone PCR by changing Mg^{+2} ion concentrations or other reaction conditions, it is possible to introduce random mutations at the rate of 0.2–3.0 per 100 base pairs. Reetz et al. [25] used the extracellular lipase from *Pseudomonas aeruginosa* (PAL) to demonstrate the principle of creating an enantioselective enzyme by random mutagenesis. The native PAL enzyme has no enantioselectivity in the hydrolysis of racemic 2-methyldecanoic acid *p*-nitrophenyl esters (Fig. 7). In just four rounds of mutagenesis using error-prone PCR, the enantioselectivity was increased from 2% enantiomer excess (ee) to 31%, 57%, 75% and 81% ee, respectively, in favor of the (*S*)-configured acid. This random mutagenesis approach has identified amino acid positions related to enantioselectivity without knowledge of the 3-D structure of the PAL enzyme. With two more rounds of site-specific saturated mutagenesis, introducing all amino acids at those important amino acid positions related to enantioselectivity, Liebeton et al. [26] further improved the enantioselectivity of the PAL enzyme to 90% ee.

Gene domain shuffling Stemmer [27] reported an in vitro recombination method that involves digesting a gene family carrying different mutations with DNase I to a pool of random DNA fragments (Fig. 8). These fragments can be reassembled into a full-length gene by repeated cycles of annealing and DNA replication using PCR. The fragments prime each other, based on homology, and recombination occurs when fragments from one copy of gene prime onto another copy, causing template switching. This method can generate a library of chimeric genes and subsequent HTS can identify the desired mutants. Using this method, Crameri et al. [28] demonstrated that, using four cephalosporinase genes from different bacterial species, a single cycle of shuffling yielded a 270- to 540-fold improvement in hydrolytic activity towards Moxalactam. The best clone contained eight segments from three of the four genes and had 33 amino acid point mutations. Molecular breeding by gene shuffling can efficiently mix sequences from different species and is a powerful tool to accelerate the accumulation of beneficial mutations, compared with the random mutagenesis method.

One of the deficiencies of the above method is that, when it is applied to regions of low sequence homology, recombination is relatively inefficient and only a small number of variants result. Coco et al. [29] presented an improved method termed ''random chimeragenesis on transient templates'', or RACHITT. This method is distinct from the Stemmer's [27] strategy, in that single-stranded fragments of a gene family are hybridized onto a full-length single-stranded homologous template, which servs as a scaffold (Fig. 9). The resulting full-length chimeras are fractionated, followed by treatment with nuclease, polymerase and ligase to repair the gaps. The scaffold is then removed and subsequent cycles of PCR generate a chimeric gene library. Using this strategy, Coco and colleagues demonstrated the efficiency of the RACHITT strategy by the strain improvement of

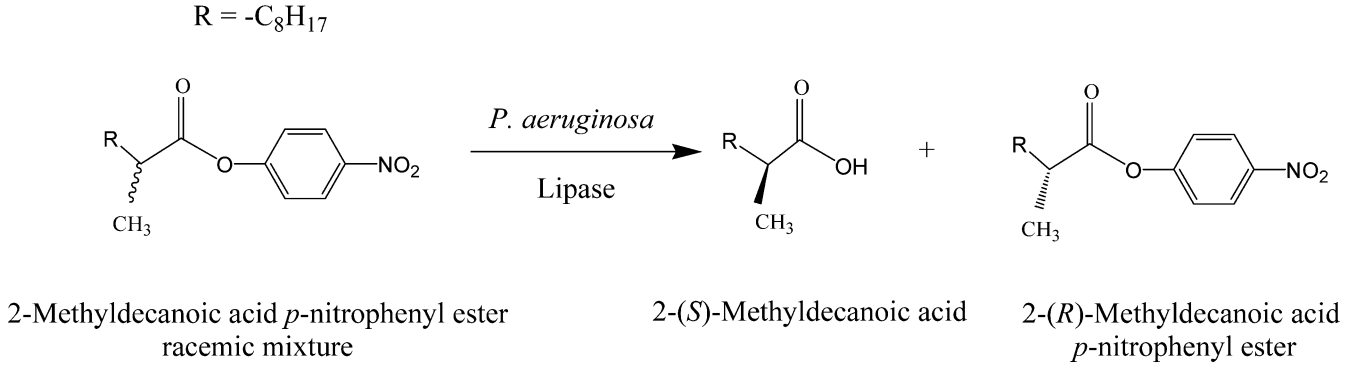


Fig. 7 Lipase-mediated enzymatic resolution of racemic esters

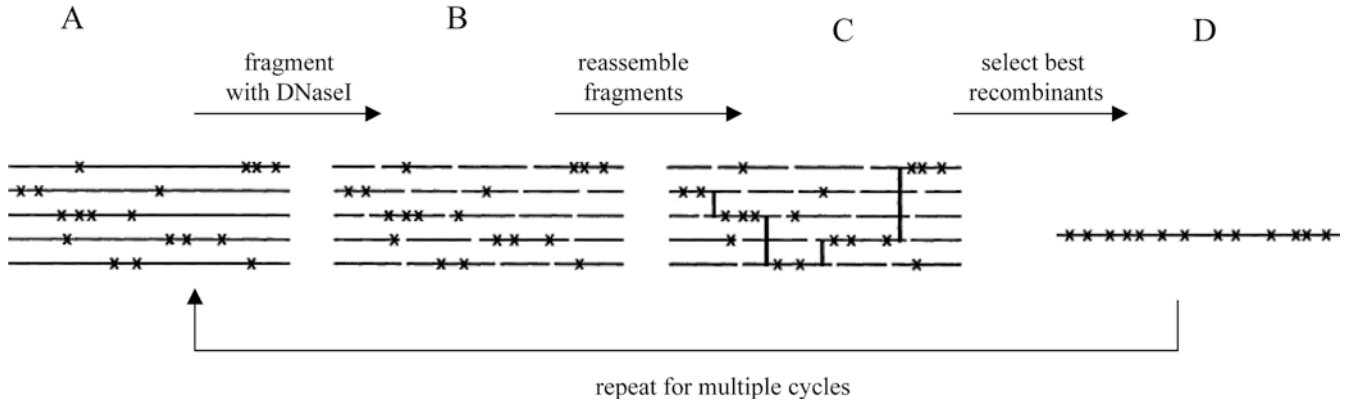


Fig. 8 Gene domain shuffling by random fragmentation and reassembly [27]. Copyright (1994) National Academy of Science, USA

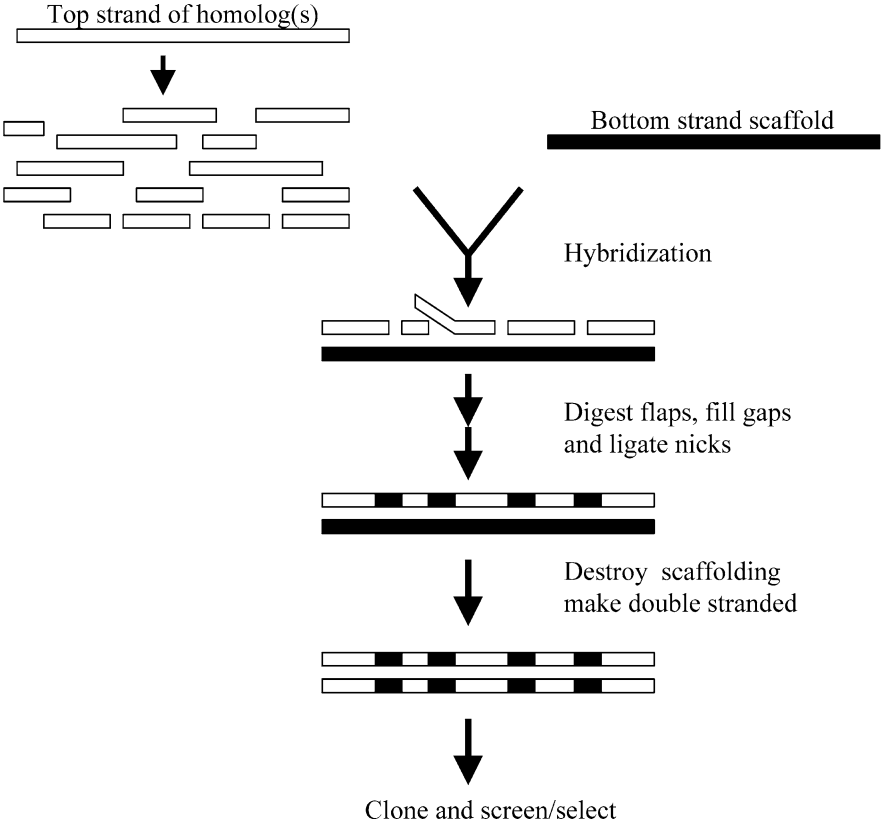


Fig. 9 Gene shuffling by RACHITT. [29], adapted, copyright (2001) Nature Publishing Co.

the *dszC* gene encoding dibenzothiophene monooxygenase, which catalyzes the first step of the diesel biodesulfurization pathway in *R. erythropolis* IGTS8. The best *dszC* mutants improved the specific monooxygenase enzyme activity three-fold and the rate of desulfurization two-fold in the *R. erythropolis* host. Moreover, this chimeric library has an average of 14 crossovers per gene, which is much higher than the Stemmer's [27] method.

Conclusion

In the past, enzyme engineering required the prior knowledge of the 3-D structure of the enzyme before any rational design of mutation could be carried out. With advances in PCR technology, random or saturated site-specific mutagenesis and gene shuffling have been demonstrated in the laboratory to be the most efficient method of generating enzyme mutants with a range of new properties: stability over a pH and temperature range, increased catalytic rate, enantioselectivity, increased substrate specificity, novel substrate activity, or resistance to organic solvents.

Using genetic engineering techniques, the objectives for enzyme engineering have been mostly achieved. However, our ability to create genetic diversity has outpaced our ability to develop HTS systems for screening the desired enzyme properties. In order to increase the throughput of an assay, the addition of a colorimetric group to a substrate or a substrate analogue is commonly used for the design of a HTS system; and enzyme activity is detected by the release of the chromophore. Using a "surrogate substrate" often results in enzymes optimized for the surrogate, but not the desired substrate. Therefore, using the desired compound in the screening system is absolutely essential for the success of finding the desired enzyme mutants. Moreover, poor substrate solubility, the incompatibility of solvents and plastic multi-well plates, the incompatibility of solvents and microbial cells and the implementation of high throughput HPLC or GC assays often limit the current HTS systems. Breakthroughs in these areas will bring more applications in the use of tailored enzymes in the area of chiral chemistry and making hybrid metabolites.

References

1. Cohen SN, Chang A, Boyer H, Helling R (1973) Construction of biologically functional bacterial plasmids *in vitro*. Proc Natl Acad Sci USA 70:3240–3244
2. Jackson DA, Symons RH, Berg P (1972) Biochemical method for inserting new genetic information into DNA of simian virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of *Escherichia coli*. Proc Natl Acad Sci USA 69:2904–2909
3. Hopwood DA, Malpartida F, Kieser HM, Ikeda H, Duncan J, Fujii I, Rudd BAM, Floss HG, Omura S (1985) Production of 'hybrid' antibiotics by genetic engineering. Nature 314:642–644
4. Miller JH, Reznikoff WS (1980) The operon. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. Fujii T, Gramajo HC, Takano E, Bibb MJ (1996) *redD* and *actII*-ORF4, pathway specific regulatory genes for antibiotic production in *Streptomyces coelicolor* A3(2), are transcribed in vitro by an RNA polymerase holoenzyme containing σ^{hrdD}. J Bacteriol 178:3402–3405
6. Wezel GP van, White J, Hoogvliet G, Bibb MJ (2000) Application of *redD*, the transcriptional activator gene of the undecylprodigiosin biosynthesis pathway, as reporter for transcriptional activity in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. J Mol Biotechnol 2:551–556
7. Demain L, Aharonowitz Y, Martin J-F (1984) Metabolic control of secondary biosynthetic pathways. In: Vining LC (ed) Biochemistry and genetic regulation of commercially important antibiotics. Addison–Wesley, Cambridge, Mass., pp 49–72
8. Basch J, Chiang S-J (1998) Genetic engineering approach to reduce undesirable by-products in cephalosporin C fermentation. J Ind Microbiol Biotechnol 20:344–353
9. Skatrud PL, Tietz AJ, Ingolia TD, Cantwell CA, Fisher, DL, Chapman JL, Queener SW (1989) Use of recombinant DNA to improve production of cephalosporin C by *Cephalosporium acremonium*. Bio/Technology 7:477–485
10. Usher JJ, Hughes DW, Lewis MA, Chiang S-J (1992) Determination of the rate-limiting step(s) in the biosynthetic pathways leading to penicillin and cephalosporin. J Ind Microbiol 10:157–163
11. Ikeda H, Omura S (1995) Control of avermectin biosynthesis in *Streptomyces avermitilis* for the selective production of a useful component. J Antibiot 48:549–562
12. Stutzman-Engwall K, Conlon S, Fedechko R, Kaczmarek F, McArthur H, Krebber A, Chen Y, Minshull J, Raillard SA, Gustafasson C (2003) Engineering the *ave*C gene to enhance the ratio of doramectin to its CHC-B2 analogue produced in *Streptomyces avermitilis*. Biotechnol Bioeng 82:359–369
13. Cantwell CA, Beckman RJ, Dotzlaf JE, Fisher DL, Skatrud PL, Yeh WH, Queener SW (1990) Cloning and expression of a hybrid *Streptomyces clavuligeruscefE* gene in *Penicillium chrysogenum*. Curr Genet 17:213–221
14. Crawford L, Stepan AM, McAda PC, Rambosek JA, Conder MJ, Vinci VA, Reeves CD (1995) Production of cephalosporin intermediates by feeding adipic acid to recombinant *Penicillium chrysogenum* strains expressing ring expansion activity. Biotechnology 13:58–62
15. Sutherland J, Bovenberg R, Lann J van der (1997) Improved process for the production of semi-synthetic cephalosporins via expandase activity on penicillin G. World patent WO 97/20,053
16. Pfeifer BA, Khosla C (2001) Biosynthesis of polyketides in heterologous hosts. Microbiol Mol Biol Rev 65:106–118
17. Chotani G, Dodge T, Hsu A, Kumar M, LaDuca R, Trimbur D, Weyler D, Sanford K (2000) The commercial production of chemicals using pathway engineering. Biochim Biophys Acta 1543:434–455
18. Gatenby AA, Haynie SL, Nagara-Jan V, Nair RV, Nakamura CE, Payne MS, Picataggio SK, Dias-Torres M, Hsu AK-H, Lareau RD, Trimbur DE, Whited GM (1998) Methods for the production of 1,3-propanediol by recombinant organisms. World patent WO 98/21,339
19. Hopwood DA (1997) Genetic contributions to understanding polyketide synthases. Chem Rev 97:2465–2497
20. Stassi DL, Kakavas SJ, Reynolds KA, Gunawardana G, Swanson S, Zeidner D, Jackson M, Liu H, Buko A, Katz L (1998) Ethyl-substituted erythromycin derivatives produced by directed metabolic engineering. Proc Natl Acad Sci USA 95:7305–7309
21. Chiang S-J, Chang LT, Chen YS, Hou HH, Elander RP (1991) Strain improvement in *Penicillium chrysogenum*, from classical genetics to genetic engineering. In: Kleinkauf H, Dohren H von (eds) 50 years of penicillin applications—history and trends. Public Press, Bratislava, pp 245–257
22. Chiang S-J, Basch J (1999) Cephalosporins. In: Flickinger MC, Drew SW (eds) Bioprocess technology: fermentation, biocatalysis and bioseparation, vol 1. Wiley, New York, pp 560–570

23. Baldwin JE, Goh K-C, Schofield CJ (1992) Oxidation of deacetylcephalosporin C by deacetoxycephalosporin C/deacetylcephalosporin C synthase. J Antibiot 45:1378–1381
24. Chiang S-J, Tonzi S, Burnett WB (1996) Penicillin V amidohydrolase gene from *Fusarium oxysporum*. US patent 5,516,679
25. Reetz MT, Zonta A, Schimossek K, Liebeton K, Jaeger K-E (1997) Creation of enantioselective biocatalysts for organic chemistry by in vitro evolution. Angew Chem Int Ed Engl 36:2830–2832
26. Liebeton K, Zonta A, Schimossek K, Nardini M, Lang D, Dijkstra BW, Reetz MT, Jaeger K-E (2000) Directed evolution of an enantioselective lipase. Chem Biol 7:709–718
27. Stemmer WPC (1994) DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. Proc Natl Acad Sci USA 91:10747–10751
28. Crameri A, Raillard S-A, Bermudez E, Stemmer WPC (1998) DNA shuffling of a family of genes from diverse species accelerates directed evolution. Nature 391:288–291
29. Coco WM, Levinson WE, Crist MJ, Kektor HJ, Darzins A, Pienkos PT, Squires CH, Monticello DJ (2001) DNA shuffling method for generating highly recombined genes and evolved enzymes. Nat Biotechnol 19:354–359