Metabolic Engineering for the Production of Natural Products

Lauren B. Pickens,[1,*] Yi Tang,[1,2] and Yit-Heng Chooi[1,*]

[1]Department of Chemical and Biomolecular Engineering and [2]Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095; email: yitang@ucla.edu

Annu. Rev. Chem. Biomol. Eng. 2011. 2:211–36

First published online as a Review in Advance on March 8, 2011

The *Annual Review of Chemical and Biomolecular Engineering* is online at chembioeng.annualreviews.org

This article's doi:
10.1146/annurev-chembioeng-061010-114209

1947-5438/11/0715-0211$20.00

*These authors contributed equally to the work.

Keywords

strain improvement, secondary metabolism, combinatorial biosynthesis

Abstract

Natural products and their derivatives play an important role in modern healthcare as frontline treatments for many diseases and as inspiration for chemically synthesized therapeutics. With advances in sequencing and recombinant DNA technology, many of the biosynthetic pathways responsible for the production of these chemically complex yet valuable compounds have been elucidated. With an ever-expanding toolkit of biosynthetic components, metabolic engineering is an increasingly powerful method to improve natural product titers and generate novel compounds. Heterologous production platforms have enabled access to pathways from difficult to culture strains, systems biology and metabolic modeling tools have resulted in increasing predictive and analytic capabilities, advances in expression systems and regulation have enabled the fine-tuning of pathways for increased efficiency, and characterization of individual pathway components has facilitated the construction of hybrid pathways for the production of new compounds. These advances in the many aspects of metabolic engineering not only have yielded fascinating scientific discoveries but also make it an increasingly viable approach for the optimization of natural product biosynthesis.

INTRODUCTION

PK: polyketide

NRP: nonribosomal peptide

PKS: polyketide synthase

NRPS: nonribosomal peptide synthetase

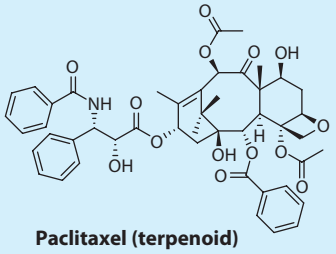
Natural products produced by plants, bacteria, and fungi have been a rich source of bioactive compounds for drug discovery and development. Natural products dominated early drug discovery as large screening programs were set up following the isolation and pharmacological demonstration of penicillin in the 1940s (1). As of 1990, 80% of drugs in use were natural products or inspired by them (2). In more recent years this figure has decreased in favor of synthetic compound libraries; however, natural products still play an important role in drug discovery. From 1981 to 2006, 52% of the new chemical entities approved by the FDA were natural products, their derivatives, or natural product analogs (3). All natural products are secondary metabolites that are not required for growth of the producing organisms. Unlike primary metabolites, which are required for growth and are mostly the same across the spectrum of living organisms, secondary metabolites can vary widely from species to species and encompass a diverse array of complex chemical structures. **Figure 1** gives an example of this diversity and highlights the major classes of natural products that are discussed in this review: terpenoids, alkaloids, polyketides (PKs), and nonribosomal peptides (NRPs). Terpenoids, or isoprenoids, are the largest class of plant metabolites and are assembled from five-carbon isoprene building blocks. These building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), are combined to form longer chain isoprenoids such as 15-carbon farnesyl diphosphate and 20-carbon geranylgeranyl diphosphate, which are utilized by the artemisinin and paclitaxel pathways, respectively. Alkaloids are another large class of plant natural products and encompass a diverse range of low-molecular-weight nitrogen-containing compounds. They are often subclassified on the basis of the nitrogen-containing precursor utilized (typically an amino acid). For example, benzylisoquinoline alkaloids (BIAs) such as berberine are derived from tyrosine, and monoterpenoid indole alkaloids (MIAs) such as camptothecin are derived from tryptophan metabolism. Biosynthetically the PK pathway is similar to the fatty acid pathway in which chain elongation proceeds via a Claisen-like condensation of acyl-coenzyme A (CoA) extender units (usually malonyl-CoA or methylmalonyl-CoA). As with fatty acid synthases, polyketide synthases (PKSs) can be large multifunctional proteins (type I PKSs) or a set of individual monofunctional proteins (type II PKSs). Type III PKSs are single proteins mainly found in plants and catalyze chain elongation with malonyl-CoA from a variety of starter units including cinnamoyl-CoA, as seen during flavonoid and stilbene biosynthesis. Finally, NRPs are produced by large multifunctional nonribosomal peptide synthetases (NRPSs), which are organized in a modular fashion similar to type I PKSs. One module is required for each amino acid incorporated into the final peptide. The reader is referred to one of the many more comprehensive texts for more in-depth treatment of these important biosynthetic pathways (4). Many of these natural products are structurally complex, as they contain multiple chiral centers and labile connectivities that make them difficult to synthesize chemically. Biosynthesis and fermentative approaches are therefore important tools in the production and development of these compounds for pharmaceutical, agricultural, and related applications (5).

Natural product discovery and process development are labor- and resource-intensive tasks. Metabolic engineering, which is the introduction of rational changes in the genetic makeup of an organism to alter the metabolic profile or improve biosynthetic capabilities (6), has gained

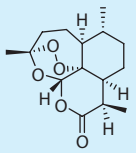
Figure 1

Major classes of natural products. PK, polyketide; NRP, nonribosomal peptide.

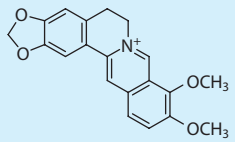
Plant natural products



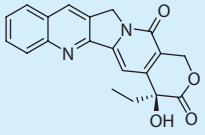
Paclitaxel (terpenoid)
Anticancer



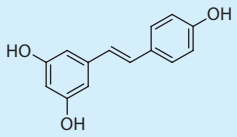
Artemisinin (terpenoid)
Antimalaria



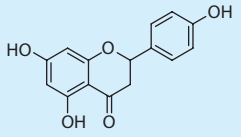
Berberine (alkaloid)
Anticancer



Camptothecin (alkaloid)
Anticancer

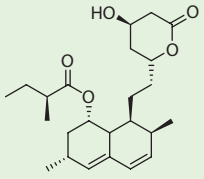


Resveratrol (PK)
Antioxidant

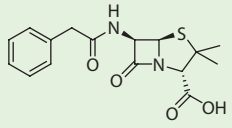


Naringenin (PK)
Antioxidant

Fungal natural products

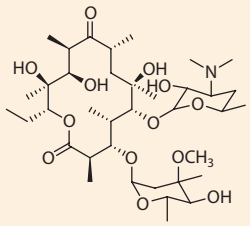


Lovastatin (PK)
Anticholesterol

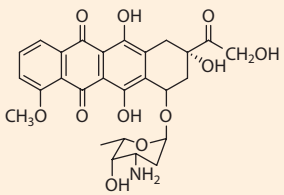


Penicillin (NRP)
Antibiotic

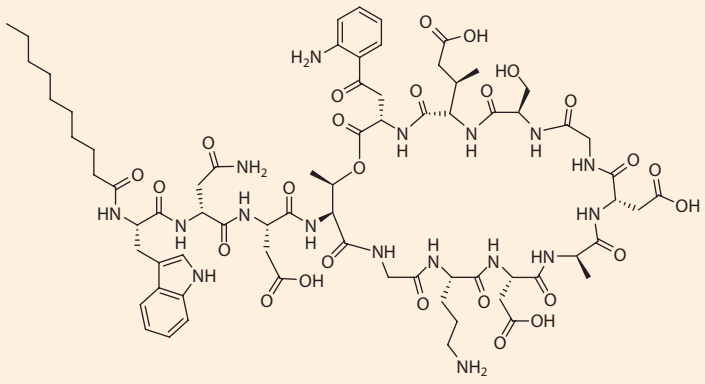
Bacterial natural products



Erythromycin A (PK)
Antibiotic



Doxorubicin (PK)
Anticancer



Daptomycin (NRP)
Antibiotic

increasing interest from researchers as a way to develop high titer bioprocesses and produce new "nonnatural" natural products. Advances in molecular biology techniques and knowledge of metabolic pathways have enabled unprecedented control over these complex biosynthetic processes. The dramatic decrease in the cost of sequencing technology has made available an exponential amount of genomic data and led to the identification of many genes involved in natural product pathways. Genome-scale metabolic reconstructions have been developed for many important organisms and can be used to guide metabolic engineering by identifying distal targets for genetic modification and by reducing the likelihood of unintended consequences that may not be clear from local pathway analysis alone (7). Furthermore, advances in protein engineering have led not only to improved enzyme efficiency but also to custom-tailored enzyme functions (8). Through the use of these tools and several others, metabolic engineering has been applied toward two main objectives in natural product biosynthesis: (*a*) increasing the titer of the target compound and (*b*) modifying the natural product scaffold for improved pharmacological properties. Although many of the examples are compound-specific, we discuss some general strategies used to achieve these two goals in this review.

METABOLIC ENGINEERING FOR STRAIN IMPROVEMENT

In nature, secondary metabolites are thought to have evolved to confer some selective advantage to the producing organism (9). As the production of the secondary metabolites is metabolically costly to the cell, the producing organisms have likely evolved to synthesize just enough of the secondary metabolite to have a selective advantage. However, from a biotechnological perspective, these amounts are far lower than those necessary for industrial-scale production (10). Additionally, an increasing number of cryptic pathways have been discovered that do not actively produce any metabolites. However, these pathways can be activated under certain conditions, many of which are unknown. The metabolites in these pathways therefore represent a new source of natural products (11). These factors, however, necessitate that producing organisms undergo significant strain improvement to yield the desired metabolite at an industrial scale.

Traditionally, strain improvement was achieved by screening for the highest-producing native strain and further improving the strain by rounds of mutation and selection (10). A classic example is penicillin. Intensive early screening resulted in isolation of a strain of *Penicillium chrysogenum* that produced penicillin at 100-fold higher titer than Fleming's original strain. Additional strain improvement and screening using this black box approach further increased production; industrial strains are now estimated to produce penicillin at 100,000-fold higher titer than the original strain (12). An advantage to this approach is that no knowledge of the biochemical pathways or genetics of the microorganism is needed; one simply selects the mutant strains with the best properties. This process can be time and resource intensive, however, as the probability of isolating a mutant with improved properties is estimated to be on the order of 1 in 10,000 (10). With advances in our understanding of metabolic pathways and improvements in genetic tools, rational strain improvement by metabolic engineering is a promising tool for improving natural product yield. Many genetic strategies can be used to redirect metabolic flux toward production of a desired metabolite, including, but not limited to, increasing the precursor supply, overexpressing or increasing the efficiency of bottleneck enzymes, altering the regulation of gene expression, reducing flux toward unwanted by-products or competing pathways, and reconstituting entire pathways in a heterologous host (**Figure 2**). Most successful cases utilize a combination of these strategies to achieve the best performance. This section covers methods employed for metabolic engineering of natural product pathways for improved efficiency and yield, with a focus on biosynthesis in microorganisms.

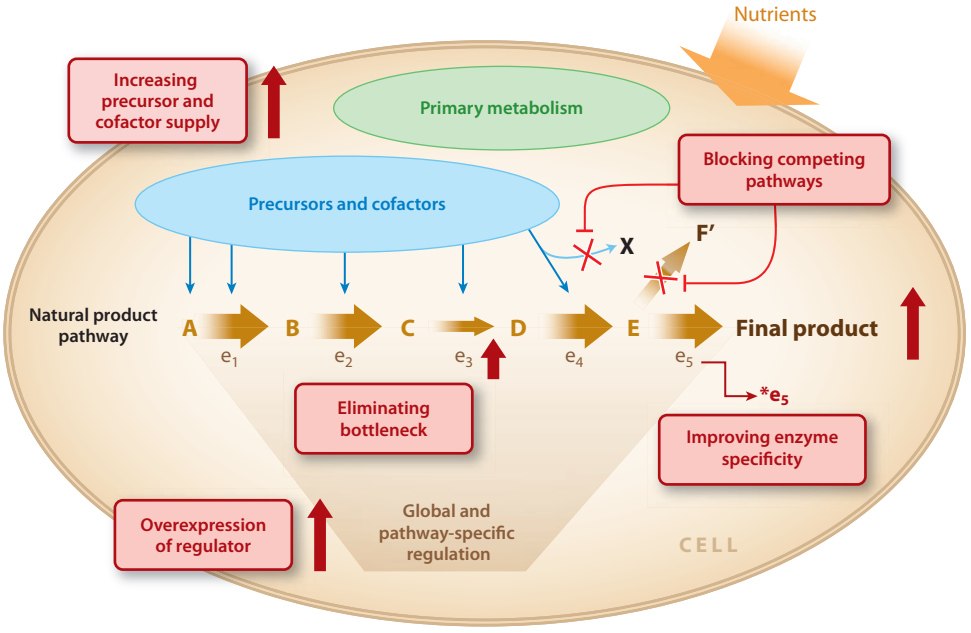


Figure 2

General metabolic engineering strategies for improvement of product titer. A–F represent intermediates in the natural product pathway, whereas e_n represent enzymes.

Biosynthesis in Heterologous Hosts

Of the strategies mentioned above, an important decision to make is whether to optimize the native producing strain or to transfer the desired pathway into a heterologous host for optimization. A heterologous host may be desirable if the native producer does not grow well under industrial fermentation conditions, is genetically intractable, or has long growth periods. The choice of a heterologous host is often determined by the source of the pathway to be transferred and the type of metabolite produced.

Natural products from bacteria. Bacteria are responsible for 70% of all known natural products produced by microorganisms. Of these, more than 75% are produced by Actinobacteria, which includes the prolific *Streptomyces* genus (13). The largest groups of natural products produced by bacteria are PKs such as erythromycin and tetracycline, NRPs such as daptomycin and vancomycin, hybrid PK/NRP antibiotics such as epothilone, and β-lactams such as cephamycin. Many actinomycetes strains have been well-characterized, and several have well-established methods for genetic modification (14). However, the growth time of actinomycetes is still slow compared with other bacteria. Additionally, not all strains perform well under industrial conditions or are genetically tractable, making heterologous hosts an attractive option in some cases.

The most common heterologous hosts for bacteria-derived natural products are either heterologous *Streptomyces* hosts (for pathways originating from Actinobacteria) or the well-characterized *Escherichia coli*. *Streptomyces* hosts are advantageous because many already produce antibiotics and therefore have the pathways for necessary precursor molecules. Additionally, *Streptomyces* bacteria are physiologically different from *E. coli*, and some proteins from *Streptomyces* pathways have not

PPTase: phosphopantetheinyl transferase

yet been successfully expressed in *E. coli*. An example is the ketosynthase α/β heterodimer that is responsible for synthesis of the poly-β-ketone backbone during type II bacterial aromatic PK synthesis. *Streptomyces lividans* and *Streptomyces coelicolor*, among others, have been used for heterologous expression, as they have been well studied and have established protocols for genetic manipulation. One consideration when using a *Streptomyces* strain as a heterologous host is that many established strains also produce their own antibiotics, which then are in competition for building blocks or can interfere with the transplanted heterologous pathway. Several engineered strains have been constructed, including *S. coelicolor* CH999 (15) and *S. lividans* K4-114 (16), in which the native antibiotic pathways are knocked out to create a clean background for production of a new compound. For example, heterologous production and ease of purification of the NRP daptomycin in *S. lividans* were increased upon elimination of the actinorhodin-producing pathway (17). Another strategy is to select an industrial strain that has already been optimized through mutation and introduce a pathway of interest. For example, recently Reynolds and coworkers (18) utilized an industrial monesin producer, *Streptomyces cinnamonensis*, to produce the PK antibiotic tetracenomycin in titers significantly higher than for the native producer. Many of the mutations introduced by the industrial strain development process are expected to be in genes outside the antibiotic pathway and may be difficult to predict. Therefore, starting with an already optimized strain may be a shortcut to increasing yield of a product composed of similar building blocks.

Despite these advantages of actinomycetes hosts, *E. coli* still offers a much shorter growth time and easier genetic manipulations. Many bacterial natural product pathways have been successfully expressed in *E. coli*, including type I modular PKSs (19–21), NRPSs (22, 23), and hybrid PKS/NRPS systems (24, 25). The first successful example of heterologous expression of a complex PK pathway in *E. coli* was erythromycin (19). Erythromycin is produced by a type I modular PKS consisting of three multidomain subunits, 6-deoxyerythronolide synthase (DEBS) 1 (370 kDa), DEBS 2 (380 kDa), and DEBS 3 (332 kDa), which together produce the macrolide core 6-deoxyerythronolide B (6-dEB). One consideration when using *E. coli* as a host is that not all of the necessary biosynthetic components or substrates may be available. For PK biosynthesis, the PKS must be posttranslationally modified by a phosphopantetheinyl transferase (PPTase). To achieve this, the authors (19) inserted the *sfp* PPTase gene into the *E. coli* chromosome. Additionally, *E. coli* does not produce the (2*S*)-methylmalonyl-CoA extender unit required by DEBS; therefore, two genes, *pccA* and *B*, from *S. coelicolor* were transferred to *E. coli* to convert propionyl-CoA to (2*S*)-methylmalonyl-CoA. Further modifications to increase the efficiency of the system included removing the propionate catabolism pathway and upregulating propionyl-CoA ligase and biotin ligase to enhance precursor supply. The resulting strain produced 6-dEB at a rate of 0.1 mmol per gram of cellular protein per day (19). Later the completely tailored erythromycin C was produced in *E. coli* by adding genes encoding the deoxysugar biosynthetic pathway and tailoring enzymes needed to convert 6-dEB to erythromycin C, along with the gene to confer erythromycin resistance. This resulted in successful production of erythromycin C, although at a much lower yield than for the native producer (21). Like PKSs, NRPSs also require posttranslational pantetheinylation of the individual thiolation domains to be fully functional. Watanabe and coworkers (23) achieved total biosynthesis of the antitumor NRP echinomycin by introducing a three-plasmid system carrying the echinomycin biosynthetic genes from *Streptomyces lasaliensis*; *sfp*; *fabC*, which encodes fatty acid acyl carrier protein and a gene conferring echinomycin resistance in *E. coli*. Although currently these strains do not outcompete production in the native hosts, further strain improvement combined with the advantages of working with *E. coli* may make this a viable system for future production of bacterial natural products and engineered analogs. Other reviews elaborate further on heterologous biosynthesis of bacterial natural products (26–29).

Natural products from fungi. Fungi are responsible for approximately 30% of known microbial natural products, a share that is on the rise owing to increasing interest in fungal natural products (13). Major classes of fungal natural products are PKs, peptide-based compounds such as NRPs and β-lactams, terpenoids, and combinations of these (30). Many of these are medicinally important, such as the blockbuster cholesterol-lowering drug lovastatin produced by a set of PKSs, the well-known β-lactams penicillin and cephalosporin, and the cyclic peptide immunosuppressant cyclosporine. Heterologous expression of these important metabolites has, however, not yet been utilized to the same extent as that of bacterial natural products (30). Many fungi are already good hosts for secondary metabolite production, as they grow well on cheap carbon sources, and researchers have had great success with traditional strain and process improvement strategies, which have resulted in multiple grams per liter titers of the desired pharmaceutical products (31).

An additional difficulty for heterologous expression of fungal gene clusters is the mRNA processing that may be required to produce functional protein. This makes heterologous fungal strains an attractive option, as one can bypass the time-consuming task of removing introns and stitching genes by the polymerase chain reaction that is required to ensure correct expression in *E. coli* and yeast (32). Like the actinomycetes, several of the more robust, well-characterized fungal strains may be good candidates for heterologous hosts for gene clusters that originate from more recalcitrant strains. The first metabolite produced by a fungal synthase in a heterologous host was 6-methylsalicylic acid, which is synthesized by the multifunctional PKS 6-methylsalicylic acid synthase (6-MSAS). Successful heterologous expression was achieved in *S. coelicolor* CH999 (33), *E. coli* (34), *Saccharomyces cerevisiae* (34), and *Aspergillus nidulans* (35). In all but the *A. nidulans* host, the introns were removed from the gene encoding 6-MSAS. Without any further optimization, yields from bacteria were approximately 60–75 mg liter^{-1} (33, 34), whereas production in *A. nidulans* was more than 300 mg liter^{-1} (35), and yeast produced 1.7 g liter^{-1} (twice the production of native producer *Penicillium patulum*) (34). More recent examples in *E. coli* and fungal hosts include the heterologous production of beauvericin in *E. coli* (36) and reconstruction of the four-gene pathway to produce tenellin in *Aspergillus oryzae* (37). Heterologous hosts have also been used to express fungal synthases for in vitro study. For example, the highly reducing iterative PKS LovB from the lovastatin pathway was recently expressed and purified from an engineered *S. cerevisiae* strain (38). Although heterologous expression of fungal metabolites is still in the development stage, these successes demonstrate that heterologous hosts are a viable option for the overproduction of fungal metabolites. For further details, the reader is referred to additional reviews on this topic (32, 39, 40).

Natural products from plants. Plants too are prolific producers of many important pharmacologically active molecules including terpenoids such as artemisinin and paclitaxel, alkaloids such as camptothecin, and PKs such as flavonoids. Due to the long growth time, low yield, and environmental consequences of harvesting large volumes of plant biomass, there is a significant incentive to produce these valuable compounds in microbial hosts. The higher complexity of plants, however, introduces additional challenges. Arguably the two most difficult challenges to overcome for heterologous production of plant natural products in microbes are identifying all of the scattered genes responsible for biosynthesis of the target compound and achieving functional protein expression in the lower organism (a particular problem with plant cytochrome P450 tailoring enzymes). Despite these factors, several plant metabolites have been produced successfully in both *E. coli* and yeast, including precursors to the medicinally important terpenoids paclitaxel (41–43) and artemisinin (44, 45) as well as the alkaloid intermediate reticuline (46, 47).

Plant aromatic PKs, such as the flavonoids and stilbenes, have also been successfully produced in *E. coli* hosts using an artificial pathway construction approach in which genes from different pathways in different plants were combined to reconstruct a functional pathway (48).

MEP: 2*C*-methyl-D-erythrotol-4-phosphate

MVA: mevalonate

For example, for the biosynthesis of the flavanone naringenin in *E. coli*, the authors (49) assembled a pathway consisting of a phenylalanine ammonia-lyase from the yeast *Rhodotorula rubra*, a cinnamate/coumarate:CoA ligase from *S. coelicolor*, a chalcone synthase from the licorice plant *Glycyrrhiza echinata*, and chalcone isomerase from *Pueraria lobata*. This hybrid pathway produced naringenin at 57 mg liter^{-1}.

Additionally, some progress has been made to address the problem of plant-derived cytochrome P450 expression (50). Several groups have had success in improving P450 expression in *E. coli* using strategies such as modifying the N-terminal membrane recognition domain (51, 52). Once the target compound can be produced, one can take advantage of the range of tools and models available for well-characterized hosts such as *E. coli* and *S. cerevisiae*. Therefore, the choice of a heterologous host for any system should take into account both the host's native or initial ability to produce the target and the ease or feasibility with which it can be engineered for increased yield.

Increasing Precursor Supply

The technique of increasing precursor supply has been successfully used for most of the major classes of natural products and can be applied to both native and heterologous producing strains. Precursor supply is at the intersection of primary and secondary metabolism, as these precursors are either primary metabolites or derived from them. For example, malonyl-CoA, an important building block for the biosynthesis of PKs, is produced by carboxylation of acetyl-CoA by the acetyl-CoA carboxylase complex (ACCase). Ryu and coworkers (53) engineered *S. coelicolor* for increased production of malonyl-CoA by overexpressing the genes encoding ACCase. The result was a sixfold increase in actinorhodin production. Zha and coworkers (54) combined several strategies with overexpression of ACCase to increase malonyl-CoA levels in *E. coli*. These included overexpression of the genes encoding ACCase and acetyl-CoA synthetase, knockout of competing pathways, and elimination of malonyl-CoA degradation pathways and yielded a 15-fold increase in intracellular malonyl-CoA. Fowler et al. (55) utilized a model-based approach to identify and verify new genetic manipulations to increase flux to malonyl-CoA, which resulted in enhanced production of flavonoids in *E. coli*. In addition to malonyl-CoA, type I PKs utilize a variety of acyl-CoA extender units such as methylmalonyl-CoA, ethylmalonyl-CoA, and isobutyryl-CoA. Gene inactivation and overexpression strategies have also been used to increase the supply of these building blocks for an increase in desired PK yield (56–58).

Terpenoid-producing pathways utilize IPP as a building block to generate the longer terpenoid backbones of geranyl pyrophosphate, farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate precursors to terpenoid natural products. IPP is produced predominantly by the 2*C*-methyl-D-erythrotol-4-phosphate (MEP) or nonmevalonate pathway in prokaryotes and the chloroplasts of higher plants, and by the mevalonate (MVA) pathway in eukaryotes and the cytosol of higher plants (59). Precursor engineering strategies in *E. coli* have focused on either improving production of the endogenous MEP pathway or introduction of a heterologous MVA pathway for increased IPP production. Engineering efforts focused on the MEP pathway have found that overexpression of the genes encoding 1-deoxy-D-xylulose-5-phosphate synthase (*dxs*), 1-deoxy-D-xylulose-5-phosphate reductase (*dxr*), and/or isopentenyl diphosphate isomerase (*idi*) resulted in increased isoprenoid production (60–62). Martin and coworkers (44) bypassed the native pathway and instead introduced the MVA pathway from *S. cerevisiae* into *E. coli*, which resulted in greatly increased production of isoprenoid precursors. Further optimization by balancing individual enzyme expression relieved growth inhibition caused by accumulation of the toxic 3-hydroxy-3-methyl-glutaryl-CoA intermediate, and resulted in a further increase in isoprenoid production (63). In yeast, the flux through the native MVA pathway was increased by chromosomal integration

of an engineered form of 3-hydroxy-3-methylglutaryl-CoA reductase, a bottleneck in the MVA pathway, which resulted in a fivefold increase in amorphadiene production (45). Further increases were achieved by increasing the availability of acetyl-CoA, the primary metabolite starting point of the MVA pathway, by overexpressing acetylaldehyde dehydrogenase and introducing an additional heterologous acetyl-CoA synthetase, thereby increasing flux from pyruvate to acetyl-CoA (64).

AT: acyltransferase

The shikimate pathway is a vital part of the primary metabolism that provides precursors for aromatic amino acid biosynthesis. Several classes of natural products utilize aromatic amino acids or other metabolites derived from the shikimate pathway as precursors including flavonoids, alkaloids, and NRPs (65). Increasing flux through the shikimate pathway or downstream amino acid tailoring steps has been used successfully to increase natural product yields. For example, balhimycin is a glycopeptide antibiotic that shares the same seven–amino acid backbone as the pharmaceutically important vancomycin, differing only in its glycosylation patterns. Five of the seven amino acids that make up the aglycon are either directly or indirectly derived from the shikimate pathway. By overexpression of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase, the first enzyme in the shikimate pathway, balhimycin-specific productivity was increased by 250% (66).

Engineering Pathway-Specific Components

In the event that the product titer is not precursor limited, increasing the efficiency and expression of biosynthetic pathway enzymes may yield the greatest results. Analysis of several strains that have been classically improved by mutagenesis and screening has shown that these strains carry multiple copies of the entire gene cluster (67–69). For example, high-producing strain *P. chrysogenum* BW1890 was found to have between 8 and 16 copies of the gene cluster responsible for penicillin biosynthesis, which resulted in up to a 64-fold increase in penicillin production (68). Although this may be the simplest way for nature to evolve gene dosing strategies, this strategy may be wasteful as it increases expression of all biosynthetic enzymes rather than targeting bottlenecks in the pathway. Metabolic engineering allows one to fine-tune the expression of individual enzymes using gene dosing (70), transcriptional regulation (71), or protein engineering (72) approaches. For example, during the biosynthesis of penicillin in *A. nidulans*, it was found that aminoadipyl-cysteinyl-valine synthetase (ACVS) was the bottleneck enzyme (73, 74); however, the opposite effect was seen for *P. chrysogenum*, where the downstream acyltransferase (AT) encoded by *pen*DE was shown to be the rate-limiting enzyme (75). Another important consideration, especially for the production of bioactive natural products, is that production levels may be limited not only by biosynthetic enzymes but by the toxicity of the final compound. At higher titers the native resistance mechanisms encoded in the pathway may not be sufficient to protect the strain from the toxic effects of its own metabolite. As observed for doxorubicin biosynthesis, overexpression of resistance genes was required to obtain further improvements in product yield by alleviating product inhibition of late stage tailoring enzyme DoxA and toxic effects of doxorubicin (76–77). Therefore, there are many considerations when designing the most efficient pathway for natural product biosynthesis.

Manipulating the regulatory component of a pathway. In many organisms, pathway-specific regulators may be utilized to enhance production of the resulting natural product. For example, many gene clusters identified in *Streptomyces* encode a *Streptomyces* antibiotic regulatory protein (SARP) that has been shown to be a positive regulator of antibiotic production (78). Overexpression of the SARP from the fredericamycin-producing gene cluster of *Streptomyces griseus* ATCC 49344 resulted in a nearly sixfold increase in antibiotic production over the native strain (79). Overexpression of the SARP MtmR from the mithramycin gene cluster in *Streptomyces argillaceus* resulted

in a 16-fold increase of mithramycin titer. Interestingly, in the same study the authors found that MtmR also was able to activate the actinorhodin-producing pathway when heterologously expressed in *S. coelicolor* (80). Through the use of a similar approach in fungi, after transferring the entire citrinin biosynthetic gene cluster from native host *Monascus purpureus* to *A. oryzae*, overexpression of activator CtnA resulted in a 400-fold improvement over the heterologous strain, which carried only a single copy of *ctnA* (81). This also indicates the importance of the native regulator when transferring from one organism to another an entire gene cluster that retains the original promoters and control elements. This strategy has also been used to turn on cryptic pathways in fungi (11) and has led to the identification of two novel metabolites, aspyridone and asperfuranone, from *A. nidulans* (82, 83).

Regulation of secondary metabolite pathways can also include negative regulation by pathway-specific repressors. The gene cluster responsible for the biosynthesis of the antibiotics platensimycin and platencin from *Streptomyces platensis* MA7327 contains a GntR family transcriptional repressor. By inactivating the gene encoding this transcriptional repressor, Smanski and coworkers (84) improved the titer of platensimycin and platencin by 100-fold over the wild-type strain.

Tunable control of metabolic pathway components. When assembling a metabolic pathway one gene at a time, there are additional opportunities to modulate gene expression at the individual gene level. In addition to varying copy number, the ability to control relative amounts of individual enzymes at the transcriptional and translational levels may be a promising approach to fine-tune metabolic pathways. Promoter libraries have been developed for both *E. coli* (85) and yeast (86) to give a quantified range of expression levels. In prokaryotic systems such as *E. coli*, pathway genes are often arranged as an operon under the control of a single promoter; therefore, additional control mechanisms may be useful. Pfleger and coworkers (87) developed a library of tunable intergenic regions that control expression levels using differences in mRNA secondary structures, RNase cleavage sites, and ribosome binding site-sequestering sequences. Recently, the Voigt group (88) developed a method for predictable control of protein expression based on the design of RBS sequences. As mentioned earlier, finding the proper balance of enzyme levels was key to reducing the toxicity observed during amorphadiene production in *E. coli* (63). Determining the proper enzyme levels, however, is an additional challenge. In the heterologous production of benzylisoquinoline alkaloids in *S. cerevisiae*, Hawkins & Smolke (47) first utilized an inducible promoter system to titrate gene expression one by one and determine optimal expression levels for each gene. These optimal levels were then correlated to expression levels achieved by members of the library of mutant translation and elongation factor 1 promoters developed by Nevoigt et al. (86) to construct a new strain with engineered promoters chosen to reflect the optimized enzyme expression levels (47). Ajikumar and coworkers (43) also utilized an inducible promoter system to simultaneously optimize upstream and downstream modules of the taxadiene pathway in a multivariate approach to identify the most efficient expression levels. The resulting titer of 1 g liter^{-1} taxadiene produced from an *E. coli* host was a significant improvement upon previously reported titers of 10 mg liter^{-1} of this key paclitaxol precursor produced by *E. coli*. Another approach to determine imbalances in enzyme levels is to utilize "-omics" data to determine effects that may not be immediately obvious from observing titer alone. For example, Kizer and coworkers (89) utilized a transcriptomics and metabolomics approach to identify and ameliorate an imbalance in carbon flux during isoprenoid biosynthesis.

Protein engineering. For enzymes with low turnover or poor expression, simply overexpressing the protein may not be sufficient to generate high levels of product. Therefore, improving the enzyme through evolutionary or rational engineering methods may be desirable to increase

the efficiency of the pathway. As discussed earlier, plant cytochrome P450s have been the target of protein engineering efforts owing to the poor expression and activity of the native enzymes in *E. coli* and other heterologous hosts; these efforts have resulted in increased production of the tailored final product. Leonard & Koffas (51) designed a chimeric bidomain P450 with a plant cytochrome P450 from *Glycine max* tethered to a cytochrome P450 reductase (CPR) from *Catharansus roseus* to imitate the architecture of the efficient, bifunctional bacterial P450 BM-3. The bifunctional protein alone had low conversion in vivo; however, further optimization of the leader sequence and N-terminal membrane anchor resulted in higher flavonone conversion than the wild-type P450/CPR pair expressed in yeast (51). Chang et al. (52) also successfully expressed a terpenoid P450 hydroxylase in *E. coli* by modification of the N-terminal membrane anchor and optimization of the CPR redox partner. In another case of rational engineering, homology-guided point mutation was used to alter the active site of levopimaradiene synthase for improved productivity (72). Directed evolution is also a powerful tool to improve enzyme activity (90). The pigmented carotenoid lycopene has been used successfully in several cases as a screening mechanism for directed evolution of enzymes involved in terpene precursor biosynthesis to develop a strain capable of higher terpenoid production (72, 91).

Deletion of Competing Pathways

In addition to increasing enzyme activity, it may also be useful to downregulate or delete certain genes to eliminate competing pathways that may siphon off important precursors or intermediates, or simply contribute to an unnecessary use of cellular resources. Several examples were mentioned earlier with regard to increasing precursor supply by eliminating pathways competing for primary metabolites. For example, the endogenous yeast squalene synthase encoded by *erg9* also utilizes the sesquiterpene precursor FPP used by amorphadiene synthase. Thus, knocking out the competing *erg9* gene resulted in an increase in amorphadiene synthesis (92). During in vivo bioconversion of the lovastatin intermediate monacolin J to simvastatin using *E. coli* expressing the heterologous AT LovD, *E. coli* was found, unexpectedly, to hydrolyze the synthetic thioester substrate (93). The responsible hydrolase, BioH, was then knocked out to improve simvastatin production (93). In addition, during doxorubicin biosynthesis, several genes encoded by the *dxr* gene cluster were deleted to improve titer, as the corresponding protein products converted doxorubicin to less active derivatives (94).

In addition to obvious targets that directly act on natural product intermediates, gene deletion may be used to make the heterologous system more efficient, for example by balancing redox cofactors. Many natural product pathways encode NADPH-dependent enzymes such as oxidoreductases that may place a metabolic burden on the cell as flux through the pathway is increased. Chemler et al. (95) identified NADPH availability as a factor limiting high production of flavonoid (+)-catechins in *E. coli*. Utilizing a metabolic modeling approach, the authors identified combinations of gene knockouts to increase intracellular NADPH availability and increase flavonoid production by two- to fourfold. Due to the involvement of NADP(H) in many cellular enzymatic reactions, the effective solution space would be impossible to probe experimentally, which makes systems biology an important tool to guide metabolic engineering strategies.

An extension of the gene deletion strategy is the idea of creating a genome-minimized host for the production of secondary metabolites. The idea is that by deleting nonessential genes one can increase efficiency by directing cellular resources toward only those pathways that are necessary for survival and product biosynthesis. *E. coli* has been the main target of genome minimization efforts, which have resulted in strains with genome size reduced up to 22% from the wild type with no growth deficiency (30% reduction achieved with growth deficiency) and improved genome

KR: ketoreductase

stability and production of target metabolites (96). Komatsu and coworkers (97) recently reported a genome-minimized *Streptomyces* host. The genome of antibiotic producer *Streptomyces avermilitis* was reduced to 83% of its original size, and upon introduction of the streptomycin gene cluster, the reduced strain produced higher titers than both the parent *S. avermilitis* carrying the heterologous gene cluster and the native streptomycin producer *Streptomyces griseus*. These reports show that genome minimization may be a feasible tactic to streamline biochemical production, but there is still much about the workings of the cell that we do not know, and large-scale deletions may come with unintended effects.

METABOLIC ENGINEERING FOR STRUCTURAL DIVERSIFICATION

Natural products have evolved over millions of years to interact with biological targets. The homology in many protein structural folds and receptors across different organisms has also allowed the exploitation of these small molecules to act on drug targets that are irrelevant to the producing organisms (98). However, these natural products are often not optimized for human drug targets and may have undesirable properties or side effects clinically (e.g., toxicity, low oral availability). Therefore, many natural product–based drugs have minor structural modifications from the original natural product scaffold that are often achieved by chemical methods. Some well-known examples are rifamycin modified to rifampicin, paclitaxel to docetaxel, lovastatin to simvastatin, and geldanamycin to 17-DMAG (17-(2-dimethylamino)ethylamino-17-demethoxygeldanamycin). These semisynthetic drugs are the results of lead optimization in drug development programs; an identified natural product with biological activity (lead compound) is used as a starting point for various chemical modifications to enhance its potency, selectivity, pharmacokinetic parameters, and other desirable properties. There is increasing interest in the application of metabolic engineering as a complementary tool in lead optimization (99). In cases in which the natural products are highly complex, metabolic engineering may allow selective modifications of natural product scaffolds that may be difficult to achieve by chemical means alone. Metabolic engineering can also provide an alternative way to manufacture existing semisynthetic drugs. A classical example is the production of the clinical agent epirubicin by a single fermentation process using engineered *Streptomyces peucetius*, which eliminates the need for postfermentation processing through the low-yielding semisynthetic route (100). By integrating the downstream synthetic steps into biological systems, it is possible to produce semisynthetic drugs at lower cost and using greener chemistry.

The various strategies for increasing natural product diversity by metabolic engineering are echoed in the modes of structural diversification observed in the evolution of natural product biosynthetic pathways. In nature, new structural variations are usually generated by (*a*) the deletion or recruitment of new genes in the biosynthetic pathway or (*b*) mutations and recombination in individual genes that result in altered activity of the enzymes (101). Metabolic engineering essentially allows us to accelerate and control the evolutionary processes to select for natural product analogs with useful biological activities. We focus here on common metabolic engineering strategies useful for exploring the chemical space around bioactive natural product scaffolds and their potential application in drug discovery and development.

Gene Disruption and Mutasynthesis

One of the simplest strategies for introducing a structural change is disruption of a particular gene that acts downstream in a pathway (usually a tailoring enzyme). A typical example is the generation of a macrolide analog without the epoxide functional group (4,5-deepoxypimaricin) by disruption of a P450 epoxidase, PimD, in the pimaricin gene cluster (102). Disruption of a ketoreductase (KR)

gene in mithramycin pathway led to isolation of three analogs, including mithramycin SK, which had an improved therapeutic index compared with the parent drug (103). Two recent examples illustrate the application of targeted gene disruption to generate derivatives of pactamycin (104) and tautomycin (105) that are useful for structure-activity relationship (SAR) study. Significantly, one study used this approach to inactivate a monooxygenase gene in the pathway of the Hsp90 inhibitor macbecin to generate an analog that does not contain the benzoquinone toxicophore (106). The novel nonquinone macbecin analog has almost 100-fold improvement in Hsp90 binding affinity and significantly reduced toxicity.

SAR: structure-activity relationship

Structural variation can also be introduced by inactivation of individual domains within multidomain modular megasynthases such as PKSs and NRPSs. KR domain inactivation of PKSs was used to generate analogs of epothilones, potent cytotoxic agents; a semisynthetic derivative (ixabepilone) was recently approved as a chemotherapeutic drug (107). The polyene macrolide antibiotic amphotericin B has also been subject to structural modifications by KR domain inactivation of the PKSs to generate analogs for SAR study (108). One analog, 7-oxo-amphotericin B, had good antifungal activity and lower hemolytic activity than amphotericin B.

More often, this gene inactivation strategy is coupled with precursor feeding to generate new structural analogs, a technique commonly known as mutasynthesis. Precursor feeding exploits the promiscuity of some transfer enzymes to accept similar substrates. By disruption of the gene that produces the natural substrate, a substitute (known as a mutasynthon) can be fed into the growth medium to generate new analogs. This has been successfully applied to generate new analogs for broad classes of compounds such as the macrolides (rapamycins) (109), glycopeptides (balhimycin) (110), and aminocoumarins (novobiocin/chlorobiocin) (111, 112), which illustrates the versatility of this strategy. Recently, this approach was used to generate nonbenzoquinone analogs of the Hsp90 inhibitor geldanamycin (113, 114). By removal of the biosynthetic genes for the starter unit 3-amino-5-hydroxybenzoic acid and feeding with various 3-aminobenzoic acids and related heterocycles, a chloro-substituted nonbenzoquinone analog with significantly improved therapeutic properties was identified among other geldanamycin analogs (114).

Another good example is the mutasynthesis of fluorosalinosporamide by disruption of the *S*-adenosylmethionine-dependent chlorinase (*salL*) and feeding with 5′-fluoro-5′-deoxyadenosine (5′-FDA) in the salinosporamide-producing *Salinispora tropica* (115). The resultant fluoro-substituted compound has reversible proteasome inhibitory activity, which is unlike its chloro-substituted parent salinosporamide. A fluorinase *flA* from *Streptomyces cattleya* was later inserted into the producing host to bypass 5′-FDA feeding (116).

Recently, O'Connor and coworkers (117) demonstrated that mutasynthesis by gene-silencing methods can also be employed in plants. RNA-mediated suppression of tryptamine biosynthesis in *Catharanthus roseus* followed by feeding with 5-fluorotryptamine yielded several fluoro-substituted alkaloids. A short perspective on the application of mutasynthesis in drug discovery and development is available (118).

Pathway Engineering and Combinatorial Biosynthesis

In microorganisms, the most common modes of chemical diversification are horizontal transfer of whole or partial gene clusters followed by divergence through the recruitment of new genes and the loss of others. The results of this evolution as seen today are many families of natural products with common structural cores but with assorted tailoring modifications. The aromatic PKs, the acidic lipopeptides daptomycin/A54145, the macrolides, the aminocourmarins, and the teicoplanin family of antibiotics are some examples. The homologies and divergences observed among the genes encoding the pathways have inspired scientists to reshuffle the genes and modules between

A: adenylation

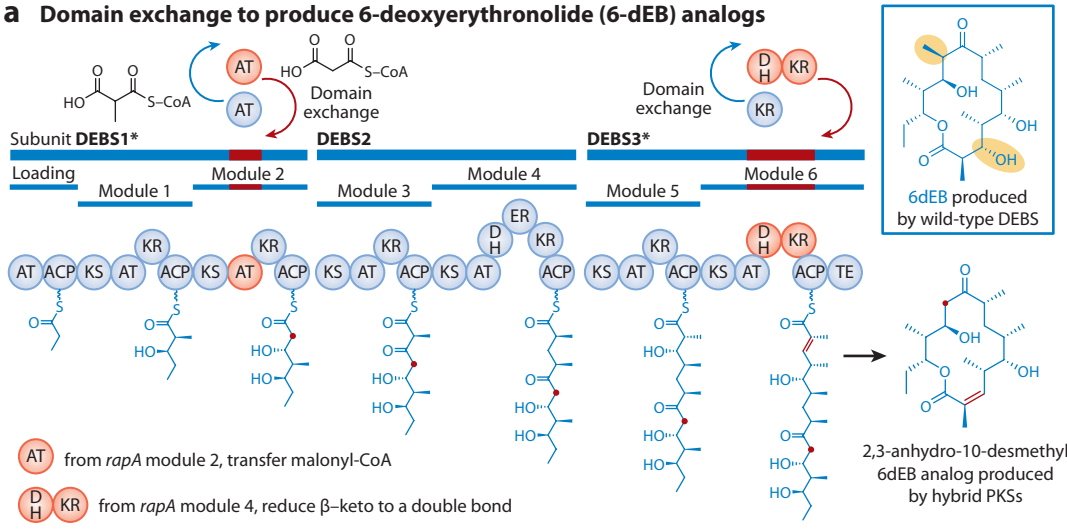
closely related natural product pathways to create new combinations. In 1985, Hopwood and coworkers (119) first demonstrated the feasibility of combinatorial biosynthesis using the pathway genes of three structurally related PKs. By the introduction of genes from the actinorhodin pathway in *S. coelicolor* into medermycin- and dihydrogranaticin-producing *Streptomyces* species, two new hybrid compounds, mederrhodin and dihydrogranatihordin, were produced.

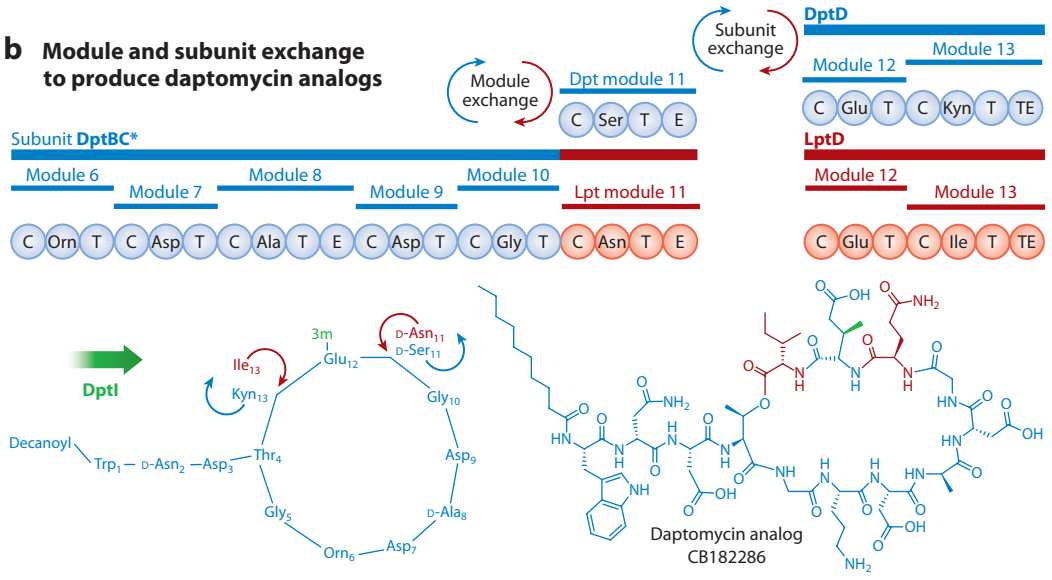
A pathway often can be manipulated to yield new product analogs by changing the combination of genes (or part of the genes) involved in the biosynthesis, provided that the enzymes downstream of the alteration point have sufficient promiscuity to tolerate the changes in the substrate (120). This implies that introducing a change in the later steps of a pathway often has a higher success rate because fewer downstream enzymes are involved. Downstream enzymes sometimes can tolerate minor alterations at early points in the pathway as long as such changes do not disrupt the overall core scaffold. Because the biosynthetic enzymes for a family of related molecules act on similar substrates or intermediates, the chances of successfully producing chimeric molecules are considerably higher when swapping genes within a family. This highlights the importance of continuous discovery and elucidation of new natural product pathways, and genome mining has emerged as a potential tool for this application (121). Metabolic pathway engineering can be performed in the native host by iterative gene deletion and introduction of new genes, or in an appropriate heterologous host by reconstitution of the combinatorial biosynthetic pathway. The use of a heterologous host is preferred when the native host is slow-growing or cannot be easily manipulated genetically (99, 122). The biosynthetic pathways can be first assembled or modified in a cloning host, such as in *E. coli*, and then the whole engineered pathway introduced into an appropriate heterologous host.

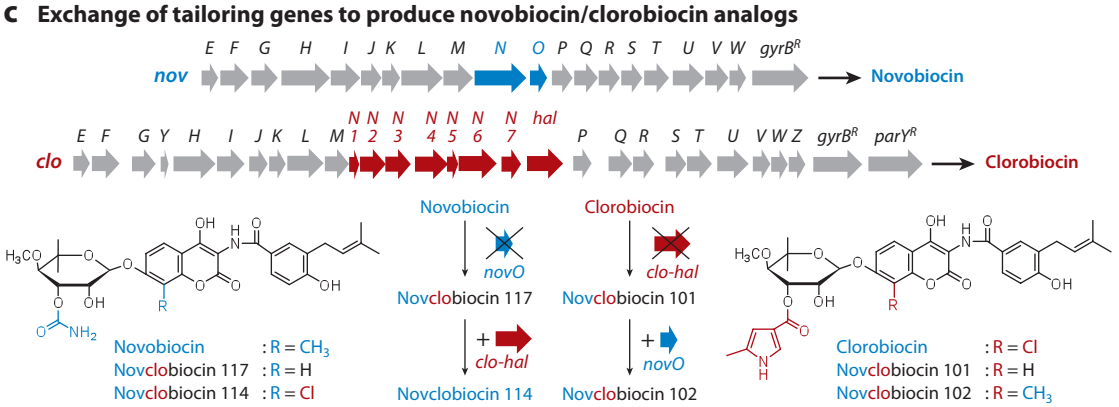
Engineering modular megasynthases. Bacterial PKSs and NRPSs are large modular enzymes that catalyze multiple reactions in an assembly-line fashion. The modularity and general colinearity of PKSs and NRPSs make them highly evolvable in nature, at both the module and domain levels (123). A good example is the iturin family of NRPs. From the structures of iturin A, bacillomycin D, mycosubtilin, and their corresponding NRPS genes, the variation in the lower half of these molecules is clearly the result of mutation and intergenic rearrangement of adenylation (A) domains in the NRPSs (124). The three 16-membered macrolides (tylosin, spiramycin and chalcomycin) also appear to have diverged from a common ancestral pathway, as their corresponding gene clusters share five similar PKS subunits (125). These modular megasynthases are particularly amenable to genetic engineering, and the pathways of these structurally related scaffolds provide a valuable toolbox for combinatorial biosynthesis. Structural modification can be achieved by mixing and matching the megasynthases at the subunit, module, and domain levels (**Figure 3*a*,*b***). The

Figure 3

Examples of pathway engineering and combinatorial biosynthesis to generate new analogs: (*a*) Exchange of 6-deoxyerythronolide synthase (DEBS) AT2 and KR6 domains with *rap*AT2 and *rap*DH/KR4 domains from rapamycin synthase (RAPS) (126). ACP, acyl carrier protein; AT, acyltransferase; CoA, coenzyme A; DH, dehydratase; ER, enoyl reductase; KR, ketoreductase; KS, ketosynthase; PKS, polyketide synthase; TE, thioesterase. Asterisks indicate chimeric proteins generated from domain or module swapping. (*b*) Exchange of daptomycin Dpt module 11 and subunit DptD with the corresponding module and subunit from the A54145 pathway (LptABCD) (131). Loading module and subunit DptA are not shown, as both were unchanged, and the parent daptomycin structure is shown in **Figure 1**. The adenylation domain is represented by the corresponding three-letter code of the amino acid it activates; C, condensation; T, thiolation; E, epimerase. DptI methylates the Glu_{12} residue at C3 (3m-Glu_{12}). (*c*) Swapping of tailoring genes *clo-hal* (halogenase) and *novO* (methyltransferase) between the clorobiocin and novobiocin pathways (112). The *nov* and *clo* gene clusters are shown in parallel to illustrate the homology and divergence of the two pathways. $gyrB^R$, gyrase B resistance gene; $parY^R$, topoisomerase IV resistance gene.







most-studied groups of molecules are the macrolides and the lipopeptide antibiotics, which are used as the main examples here.

In a landmark success approximately a decade ago, a group at Kosan Biosciences generated more than 50 analogs of 6-dEB by swapping the ATs and β-carbon processing domains between 6-dEB and rapamycin PKS in an engineered heterologous host (**Figure 3*a***) (126). With the discovery of the geldanamycin gene cluster (a macrolactam) in *Streptomyces hygroscopicus* (127), the researchers at Kosan Biosciences made a series of AT domain swaps with rapamycin PKSs to generate many derivatives, including one analog with a fourfold enhanced affinity for Hsp90 (128). Another significant example is the direct production of a semisynthetic drug, ivermectin, in a recombinant *S. avermitilis* strain harboring engineered PKSs. By substitution of the dehydratase and KR domains of module 2 of the avermectin PKS with the DH, enoyl reductase, and KR domains of module 13 from the rapamycin PKSs, 22,23-dihydroavermectins (ivermectins) were successfully produced (129), which provided a route for producing the drug using fermentation alone. Novel analogs can also be obtained by exchanging whole PKS subunits. For example, by shuffling of the PKS subunits from three related 16-membered macrolide pathways mentioned above (tylosin, spiramycin and chalcomycin), hybrid molecules that varied in methyl, ethyl, or methoxy side chains at several positions were produced (125).

A group at Cubist Pharmaceuticals spearheaded the combinatorial biosynthesis effort to produce analogs of daptomycin, an antibiotic approved in the United States for treatment of skin and skin structure infections. The first successful production of hybrid lipopeptides was achieved by exchanging the third subunit of daptomycin NRPS (DptD) with the corresponding NRPS subunits of the structurally related A54145 and calcium-dependent antibiotic (130). Coupling of whole-subunit exchange with deletion of the Glu_{12}-methyltransferase gene (*dptI*), module exchange, and natural side chain variation has generated more than 70 new lipopeptides (**Figure 3*b***), most of which have antibacterial activities (130–132). Daptomycin and many of the analogs have poor efficacy in the treatment of community-acquired pneumonia, likely owing to sequestration in pulmonary surfactant. The latest extension of the work involved the generation of more analogs related to A54145 and daptomycin using a similar combinatorial strategy to screen for improved antibacterial activity in the presence of bovine surfactant (133). One of the new analogs obtained was highly active in the presence of surfactant, had low acute toxicity, and showed some efficacy against *Streptococcus pneumoniae* in a mouse model of pulmonary infection, thus demonstrating the potential application of this approach in drug development.

In all of the cases above, the molecules are assembled in a stepwise linear fashion followed by a macrocyclization. The engineering of the PKSs and NRPSs above altered mainly the individual starter or extension units but did not change the overall length and the final macrocyclic scaffold. Maintaining the overall core structure allows most of the downstream tailoring enzymes to process the novel hybrid molecules and thus to generate further structural diversity. For example, by combining the DEBS domain-swapping and variation in post-PKS tailoring, additional erythromycin and erythrolide analogs have been generated (134), which illustrates the power of such an approach. As the engineering of both macrolide and lipopeptide pathways has been extensively reviewed, the reader is directed to the references for further details (99, 134–138).

Combinatorial usage of pathway enzymes. Variations in tailoring genes are often responsible for the majority of the structural diversity observed for a given family of molecules. For the reasons mentioned earlier, tailoring enzymes, which typically act at the later steps in a pathway, are good candidates for combinatorial biosynthesis. The tailoring enzymes commonly encountered perform reactions such as glycosylation, acylation, oxidation, reduction, methylation, and prenylation. For example, many classes of glycosylated natural products contain variations in the deoxysugar

moieties, which are important for the biological activity of the molecules (139). Combinatorial engineering of the glycosyltransferases from different pathways has allowed the generation of arrays of analogs differing in their glycosylation patterns for several classes of natural products, such as the glycopeptides related to vancomycin and teicoplanin, macrolides related to erythromycin and tylosin, indolocarbazoles related to rebeccamycin, and the aromatic PK anthracyclines and angucyclines (139, 140). PKs are among the natural product groups that are most intensively pursued for combinatorial biosynthesis using tailoring enzymes, and the topic has been reviewed recently by Olano et al. (141)

The enzymatic toolbox available for modification of a common structural scaffold usually consists of genes encoding for the pathways of structurally related compounds. For example, the aminocoumarins novobiocin and clorobiocin share a common structural core but differ at the C-8 position of the aminocoumarin moiety (a methyl or a chlorine group) and at the 3-OH group of the deoxysugar (a carbamoyl moiety or methyl-pyrrole-2-carboxyl). By replacing the methyltransferase gene in the novobiocin pathway with the chlorinase in the clorobiocin pathway and vice versa, in addition to inactivating the AT in the clorobiocin pathway, Li & Heide (112) produced a series of new hybrid aminocoumarins (**Figure 3*c***), which provided valuable insights into the SAR of this class of gyrase and topoisomerase IV inhibitors (142). Similarly, equipped with the genetic blueprints of two structurally related indolocarbazoles (staurosporine and rebeccamycin) and tryptophan halogenases with different regioselectivity, Salas and coworkers (143) were able to generate 32 rebeccamycin analogs by altering the patterns of glycosylation, methylation, and halogenation in a heterologous *Streptomyces albus* host. Recent extension of the work has led to the identification of two hybrid indolocarbazoles that are potent and selective inhibitors of JAK2 and IKKβ kinases (144), which underscores the potential of this strategy. More recently, Hertweck and coworkers (145) used an integrated approach combining mutasynthesis (starter unit feeding), combinatorial biosynthesis (exchanging tailoring *O*-methyltransferase), and biotransformation (oxidations) to generate a focused library of 15 aureothin analogs in which a few have less cytotoxicity but improved antiproliferative activities.

Artificial pathway construction. Due to the larger genomes and the scattered nature of the pathway genes in plants, the number of completely elucidated natural product pathways is significantly lower compared with microorganisms, in which the pathway genes tend to cluster together. This limits the toolbox for metabolic engineering and combinatorial biosynthesis. Through use of the artificial pathway approach with genes from different organisms, some success in the combinatorial biosynthesis of plant metabolites has been achieved in the type III PKS pathways. Horinouchi and coworkers (48, 146, 147) divided the flavonoid biosynthetic pathway into three components: substrate synthesis, PK synthesis, and post-PKS modification. By varying these three components in separate plasmid systems and coupling with precursor-directed biosynthesis, an array of novel and known plant PKs were generated. Significantly, several of the novel stilbenes inhibited the ethoxyresorufin-*O*-deethylase activity of CYP1B1 (147). Similar combinatorial approaches can be employed in the development of artificial pathway systems for other plant metabolites, especially the alkaloids (148). An extension of artificial pathway system to a plant host has been demonstrated recently by O'Connor and coworkers (149). By integrating bacterial tryptophan halogenases into the medicinal plant *C. roseus*, the group successfully generated several novel chlorinated alkaloids.

Protein Engineering

Despite the successful examples above, researchers are often confronted by the problem that the enzymes/domains downstream in the pathway have limited tolerance to the new substrate

introduced by metabolic engineering. Protein engineering by either rational design or directed evolution has been a useful tool for improving the efficiency and stability of enzymes for biocatalysis development and titer improvement. For natural product diversification, protein engineering is an attractive tool for increasing the substrate promiscuity or changing the substrate specificity of biosynthetic enzymes. For example, directed evolution has been used by Thorson and coworkers (150, 151) to expand the substrate promiscuity of glycosyltransferases to accept alternate aglycon and sugar substrates. In another instance, O'Connor and coworkers (152) performed selective mutations at the binding pockets of strictosidine synthase, a central enzyme in plant alkaloid biosynthesis, to expand and alter the substrate specificity for mutasynthesis. The engineered strictosidine synthase was transformed into *C. roseus*, resulting in a transgenic plant cell culture that can produce a variety of unnatural alkaloid compounds with precursor feeding, thus demonstrating the utility of enzyme engineering in mutasynthesis (153).

For directed evolution, a large, high-quality library and an efficient screening strategy are essential. Schmidt-Dannert et al. (154) exploited the optical properties of colored carotenoids, which allow visual screening of a large number of enzyme variants. Directed evolution of phytoene desaturase, which synthesizes conjugated double bonds, led to isolation of a pink variant producing the fully conjugated carotenoid tetradehydrolycopene. The evolved pathway was extended to produce new cyclic carotenoids with a library of lycopene cyclase variants generated by gene shuffling. A mutant cyclase, which produces the known red, cyclic carotenoid torulene via a novel pathway, was isolated in the visual screen (154). Yoshikuni et al. (155) also showed that it is possible to engineer a promiscuous biosynthetic enzyme to produce specific products. They performed saturation mutagenesis at the active site of a promiscuous γ-humulene synthase, which cyclizes farnesyl-diphosphate into primarily γ-humulene, and searched for residues that alter product specificity (plasticity residues). Using an algorithm-assisted approach, they were able to engineer γ-humulene synthase variants that cyclize farnesyl-diphosphate specifically into seven other products. Both examples above showed that enzyme engineering is potentially useful in construction of artificial natural product pathways, as a missing enzyme in a pathway can be borrowed from a closely related promiscuous enzyme.

For modular PKS and NRPS megasynthases, simple swapping of functional domains often results in nonfunctional or heavily impaired chimeric enzymes (131, 156). The problem can be twofold: (*a*) the downstream domains are unable to accept the modified substrate, as exemplified in the attempt to alter the aromatic starter unit of rifamycin by mutasynthesis (157), and (*b*) the domain swapping disrupts the quaternary interactions between protein domains, as demonstrated in a mechanistic study of AT domain swapping (156). Using the NRPS of the siderophore enterobactin as a model, Zhou et al. (158) demonstrated that just a few rounds of directed evolution can restore and even enhance the activity of the heavily impaired chimerical enterobactin NRPS swapped with a noncognate aryl-carrier protein domain. Fischbach et al. (159) further demonstrated that limited rounds of directed evolution can improve the activity of a chimeric NRPS swapped with a heterologous A domain of the same substrate specificity as the wild type as well as with a chimeric NRPS with a heterologous A domain that activates a different substrate.

These recent studies suggest that both rational enzyme design and directed evolution can be valuable tools for increasing enzyme promiscuity to tolerate modified substrates, altering enzyme specificity to generate new structural diversity, and fine-tuning the protein interactions of exogenous domains in chimeric modular megasynthases. In nature, chemical diversity is likely to evolve in a similar way, with recruitment of a promiscuous enzyme into a new pathway or intragenic recombination, followed by evolutionary fine-tuning. The increasing use of these powerful tools in the metabolic engineering of nonnatural natural products can be foreseen, and better understanding of how natural product pathways evolved may lead to new approaches toward this goal.

PERSPECTIVE AND CONCLUDING REMARKS

Accessibility is one of the main hurdles in natural product drug discovery because many natural products are naturally produced in low yields in the native organisms, and sometimes in multiple forms, which further complicates identification, isolation, and structural elucidation. Furthermore, lead optimization of natural product scaffolds by chemical modification is usually difficult and laborious, as they often possess a variety of functional groups that require multiple protection and deprotection steps. These undesirable properties have made natural products less attractive despite their large structural diversity and high hit rates, and in the past many pharmaceutical companies have turned to combinatorial synthetic libraries as their source for drug discovery. These chemical methods, however, also have their limits, and the need for new therapeutics provides motivation to develop new methods of producing novel compounds in industrial-scale quantities.

The many examples discussed here have shown that metabolic engineering is a powerful tool that can be integrated into the natural product drug discovery and development process, from lead optimization to industrial-scale production of the molecules. Advances in our knowledge of natural product biosynthetic pathways as well as the metabolic and regulatory networks at the genomic level have allowed for rational development of new compounds and high-producing strains where traditional methods have relied on purely empirical methods. With the integration of the emerging systems and synthetic biology, the application of metabolic engineering is expected to become increasingly powerful. Through metabolic engineering, some natural products that can be obtained only in low quantities, e.g., from some plants and marine organisms, can now be produced in engineered heterologous hosts in quantities that are sufficient for industrial application.

Although traditional methods of chemical diversification and classical strain improvement remain powerful techniques, and currently outperform metabolic engineering approaches in terms of the size of combinatorial libraries produced or the titers achieved for industrial microorganisms, metabolic engineering can be a complementary approach to reduce the time needed to optimize a strain, simplify downstream chemical processing, or produce analogs that would be difficult or expensive to access by chemical methods alone. By combining all these approaches, we can more fully take advantage of the rich biochemical diversity that nature has to offer.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

Due to space constraints, we apologize for any work that we were not able to cite. Research activities from our lab in this area are supported from funds from National Institute of Health, National Science Foundation, the Camille and Henry Dreyfus foundation, the Alfred P. Sloan Foundation, and the David and Lucile Packard Foundation.

LITERATURE CITED

1. Li JWH, Vederas JC. 2009. Drug discovery and natural products: end of an era or an endless frontier? *Science* 325:161–65
2. Harvey AL. 2008. Natural products in drug discovery. *Drug Discov. Today* 13:894–901
3. Newman DJ, Cragg GM. 2007. Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod.* 70:461–77

4. Dewick PJ. 2009. *Medicinal Natural Products: A Biosynthetic Approach*. West Sussex, U.K.: Wiley. 3rd ed.
5. Demain AL, Adrio JL. 2008. Contributions of microorganisms to industrial biology. *Mol. Biotechnol.* 38:41–55
6. Stephanopoulos G, Nielsen J, Aristodou A. 1998. *Metabolic Engineering*. San Diego: Academic
7. Oberhardt MA, Palsson BO, Papin JA. 2009. Applications of genome-scale metabolic reconstructions. *Mol. Syst. Biol.* 5:320
8. Savile CK, Janey JM, Mundorff EC, Moore JC, Tam S, et al. 2010. Biocatalytic asymmetric synthesis of chiral amines from ketones applied to sitagliptin manufacture. *Science* 329:305–9
9. Challis GL, Hopwood DA. 2003. Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. *Proc. Natl. Acad. Sci. USA* 100(Suppl. 2):14555–61
10. Parekh S. 2004. Strain improvement. In *The Desk Encyclopedia of Microbiology*, ed. M Schaechter, pp. 960–73. San Diego: Elsevier Academic
11. Scherlach K, Hertweck C. 2009. Triggering cryptic natural product biosynthesis in microorganisms. *Org. Biomol. Chem.* 7:1753–60
12. Rokem JS, Lantz AE, Nielsen J. 2007. Systems biology of antibiotic production by microorganisms. *Nat. Prod. Rep.* 24:1262–87
13. Berdy J. 2005. Bioactive microbial metabolites. *J. Antibiot.* 58:1–26
14. Hopwood DA, Kieser T, Bibb M, Buttner M, Chater K. 2000. *Practical Streptomyces Genetics*. Norwich: John Innes Found.
15. McDaniel R, Ebert-Khosla S, Hopwood DA, Khosla C. 1993. Engineered biosynthesis of novel polyketides. *Science* 262:1546–50
16. Ziermann R, Betlach MC. 1999. Recombinant polyketide synthesis in *Streptomyces*: engineering of improved host strains. *Biotechniques* 26:106–10
17. Penn J, Li X, Whiting A, Latif M, Gibson T, et al. 2006. Heterologous production of daptomycin in *Streptomyces lividans*. *J. Ind. Microbiol. Biotechnol.* 33:121–28
18. Li C, Hazzard C, Florova G, Reynolds KA. 2009. High titer production of tetracenomycins by heterologous expression of the pathway in a *Streptomyces cinnamonensis* industrial monensin producer strain. *Metab. Eng.* 11:319–27
19. Pfeifer BA, Admiraal SJ, Gramajo H, Cane DE, Khosla C. 2001. Biosynthesis of complex polyketides in a metabolically engineered strain of *E. coli*. *Science* 291:1790–92
20. Watanabe K, Rude MA, Walsh CT, Khosla C. 2003. Engineered biosynthesis of an ansamycin polyketide precursor in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 100:9774–78
21. Peiru S, Menzella HG, Rodriguez E, Carney J, Gramajo H. 2005. Production of the potent antibacterial polyketide erythromycin C in *Escherichia coli*. *Appl. Environ. Microbiol.* 71:2539–47
22. Gruenewald S, Mootz HD, Stehmeier P, Stachelhaus T. 2004. In vivo production of artificial nonribosomal peptide products in the heterologous host *Escherichia coli*. *Appl. Environ. Microbiol.* 70:3282–91
23. Watanabe K, Hotta K, Praseuth AP, Koketsu K, Migita A, et al. 2006. Total biosynthesis of antitumor nonribosomal peptides in *Escherichia coli*. *Nat. Chem. Biol.* 2:423–28
24. Mutka SC, Carney JR, Liu Y, Kennedy J. 2006. Heterologous production of epothilone C and D in *Escherichia coli*. *Biochemistry* 45:1321–30
25. Pfeifer BA, Wang CCC, Walsh CT, Khosla C. 2003. Biosynthesis of yersiniabactin, a complex polyketide-nonribosomal peptide, using *Escherichia coli* as a heterologous host. *Appl. Environ. Microbiol.* 69:6698–702
26. Baltz RH. 2010. *Streptomyces* and *Saccharopolyspora* hosts for heterologous expression of secondary metabolite gene clusters. *J. Ind. Microbiol. Biotechnol.* 37:759–72
27. Boghigian BA, Pfeifer BA. 2008. Current status, strategies, and potential for the metabolic engineering of heterologous polyketides in *Escherichia coli*. *Biotechnol. Lett.* 30:1323–30
28. Challis GL. 2006. Engineering *Escherichia coli* to produce nonribosomal peptide antibiotics. *Nat. Chem. Biol.* 2:398–400
29. Zhang H, Wang Y, Pfeifer BA. 2008. Bacterial hosts for natural product production. *Mol. Pharm.* 5:212–25

30. Hoffmeister D, Keller NP. 2007. Natural products of filamentous fungi: enzymes, genes, and their regulation. *Nat. Prod. Rep.* 24:393–416
31. Manzoni M, Rollini M. 2002. Biosynthesis and biotechnological production of statins by filamentous fungi and application of these cholesterol-lowering drugs. *Appl. Microbiol. Biotechnol.* 58:555–64
32. Lubertozzi D, Keasling JD. 2009. Developing *Aspergillus* as a host for heterologous expression. *Biotechnol. Adv.* 27:53–75
33. Bedford DJ, Schweizer E, Hopwood DA, Khosla C. 1995. Expression of a functional fungal polyketide synthase in the bacterium *Streptomyces coelicolor* A3(2). *J. Bacteriol.* 177:4544–48
34. Kealey JT, Liu L, Santi DV, Betlach MC, Barr PJ. 1998. Production of a polyketide natural product in nonpolyketide-producing prokaryotic and eukaryotic hosts. *Proc. Natl. Acad. Sci. USA* 95:505–9
35. Fujii I, Ono Y, Tada H, Gomi K, Ebizuka Y, et al. 1996. Cloning of the polyketide synthase gene *atX* from *Aspergillus terreus* and its identification as the 6-methylsalicylic acid synthase gene by heterologous expression. *Mol. Gen. Genet.* 253:1–10
36. Xu Y, Orozco R, Wijeratne EMK, Gunatilaka AAL, Stock SP, et al. 2008. Biosynthesis of the cyclooligomer depsipeptide beauvericin, a virulence factor of the entomopathogenic fungus *Beauveria bassiana*. *Chem. Biol.* 15:898–907
37. Heneghan MN, Yakasai AA, Halo LM, Song Z, Bailey AM, et al. 2010. First heterologous reconstruction of a complete functional fungal biosynthetic multigene cluster. *ChemBioChem* 11:1508–12
38. Ma SM, Li JWH, Choi JW, Zhou H, Lee KKM, et al. 2009. Complete reconstitution of a highly reducing iterative polyketide synthase. *Science* 326:589–92
39. Schümann J, Hertweck C. 2006. Advances in cloning, functional analysis and heterologous expression of fungal polyketide synthase genes. *J. Biotechnol.* 124:690–703
40. Schneider P, Misiek M, Hoffmeister D. 2008. In vivo and in vitro production options for fungal secondary metabolites. *Mol. Pharm.* 5:234–42
41. Huang Q, Roessner CA, Croteau R, Scott AI. 2001. Engineering *Escherichia coli* for the synthesis of taxadiene, a key intermediate in the biosynthesis of taxol. *Bioorg. Med. Chem.* 9:2237–42
42. Engels B, Dahm P, Jennewein S. 2008. Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. *Metab. Eng.* 10:201–6
43. Ajikumar PK, Xiao W-H, Tyo KEJ, Wang Y, Simeon F, et al. 2010. Isoprenoid pathway optimization for taxol precursor overproduction in *Escherichia coli*. *Science* 330:70–74
44. Martin VJJ, Pitera DJ, Withers ST, Newman JD, Keasling JD. 2003. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat. Biotechnol.* 21:796–802
45. Ro D-K, Paradise EM, Ouellet M, Fisher KJ, Newman KL, et al. 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440:940–43
46. Minami H, Kim J-S, Ikezawa N, Takemura T, Katayama T, et al. 2008. Microbial production of plant benzylisoquinoline alkaloids. *Proc. Natl. Acad. Sci. USA* 105:7393–98
47. Hawkins KM, Smolke CD. 2008. Production of benzylisoquinoline alkaloids in *Saccharomyces cerevisiae*. *Nat. Chem. Biol.* 4:564–73
48. Horinouchi S. 2009. Combinatorial biosynthesis of plant medicinal polyketides by microorganisms. *Curr. Opin. Chem. Biol.* 13:197–204
49. Miyahisa I, Kaneko M, Funa N, Kawasaki H, Kojima H, et al. 2007. Efficient production of (2 *S*)-flavanones by *Escherichia coli* containing an artificial biosynthetic gene cluster. *Appl. Microbiol. Biotechnol.* 68:498–504
50. Gillam EMJ. 2008. Engineering cytochrome P450 enzymes. *Chem. Res. Toxicol.* 21:220–31
51. Leonard E, Koffas MAG. 2007. Engineering of artificial plant cytochrome P450 enzymes for synthesis of isoflavones by *Escherichia coli*. *Appl. Environ. Microbiol.* 73:7246–51
52. Chang MCY, Eachus RA, Trieu W, Ro D-K, Keasling JD. 2007. Engineering *Escherichia coli* for production of functionalized terpenoids using plant P450s. *Nat. Chem. Biol.* 3:274–77
53. Ryu Y-G, Butler MJ, Chater KF, Lee KJ. 2006. Engineering of primary carbohydrate metabolism for increased production of actinorhodin in *Streptomyces coelicolor*. *Appl. Environ. Microbiol.* 72:7132–39
54. Zha W, Rubin-Pitel SB, Shao Z, Zhao H. 2009. Improving cellular malonyl-CoA level in *Escherichia coli* via metabolic engineering. *Metab. Eng.* 11:192–98

55. Fowler ZL, Gikandi WW, Koffas MAG. 2009. Increased malonyl coenzyme A biosynthesis by tuning the *Escherichia coli* metabolic network and its application to flavanone production. *Appl. Environ. Microbiol.* 75:5831–39
56. Cropp A, Chen S, Liu H, Zhang W, Reynolds KA. 2001. Genetic approaches for controlling ratios of related polyketide products in fermentation processes. *J. Ind. Microbiol. Biotechnol.* 27:368–77
57. Reeves AR, Brikun IA, Cernota WH, Leach BI, Gonzalez MC, et al. 2007. Engineering of the methylmalonyl-CoA metabolite node of *Saccharopolyspora erythraea* for increased erythromycin production. *Metab. Eng.* 9:293–303
58. Han SJ, Park SW, Kim BW, Sim SJ. 2008. Selective production of epothilone B by heterologous expression of propionyl-CoA synthetase in *Sorangium cellulosum*. *J. Microbiol. Biotechnol.* 18:135–37
59. Kuzuyama T. 2002. Mevalonate and nonmevalonate pathways for the biosynthesis of isoprene units. *Biosci. Biotechnol. Biochem.* 66:1619–27
60. Farmer WR, Liao JC. 2001. Precursor balancing for metabolic engineering of lycopene production in *Escherichia coli*. *Biotechnol. Prog.* 17:57–61
61. Kajiwara S, Fraser PD, Kondo K, Misawa N. 1997. Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in *Escherichia coli*. *Biochem. J.* 324:421–26
62. Kim SW, Keasling JD. 2001. Metabolic engineering of the nonmevalonate isopentenyl diphosphate synthesis pathway in *Escherichia coli* enhances lycopene production. *Biotechnol. Bioeng.* 72:408–15
63. Pitera DJ, Paddon CJ, Newman JD, Keasling JD. 2007. Balancing a heterologous mevalonate pathway for improved isoprenoid production in *Escherichia coli*. *Metab. Eng.* 9:193–207
64. Shiba Y, Paradise EM, Kirby J, Ro D-K, Keasling JD. 2007. Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids. *Metab. Eng.* 9:160–68
65. Knaggs AR. 1999. The biosynthesis of shikimate metabolites. *Nat. Prod. Rep.* 16:525–60
66. Thykaer J, Nielsen J, Wohlleben W, Weber T, Gutknecht M, et al. 2010. Increased glycopeptide production after overexpression of shikimate pathway genes being part of the balhimycin biosynthetic gene cluster. *Metab. Eng.* 12:455–61
67. Yanai K, Murakami T, Bibb M. 2006. Amplification of the entire kanamycin biosynthetic gene cluster during empirical strain improvement of *Streptomyces kanamyceticus*. *Proc. Natl. Acad. Sci. USA* 103:9661–66
68. Smith DJ, Bull JH, Edwards J, Turner G. 1989. Amplification of the isopenicillin N synthetase gene in a strain of *Penicillium chrysogenum* producing high levels of penicillin. *Mol. Gen. Genet.* 216:492–97
69. Peschke U, Schmidt H, Zhang HZ, Piepersberg W. 1995. Molecular characterization of the lincomycin-production gene cluster of *Streptomyces lincolnensis* 78–11. *Mol. Microbiol.* 16:1137–56
70. Anthony JR, Anthony LC, Nowroozi F, Kwon G, Newman JD, et al. 2009. Optimization of the mevalonate-based isoprenoid biosynthetic pathway in *Escherichia coli* for production of the anti-malarial drug precursor amorpha-4,11-diene. *Metab. Eng.* 11:13–19
71. Keasling JD. 1999. Gene-expression tools for the metabolic engineering of bacteria. *Trends Biotechnol.* 17:452–60
72. Leonard E, Ajikumar PK, Thayer K, Xiao W-H, Mo JD, et al. 2010. Combining metabolic and protein engineering of a terpenoid biosynthetic pathway for overproduction and selectivity control. *Proc. Natl. Acad. Sci. USA* 107:13654–59
73. Kennedy J, Turner G. 1996. δ-(L-α-Aminoadipyl)-L-cysteinyl-D-valine synthetase is a rate limiting enzyme for penicillin production in *Aspergillus nidulans*. *Mol. Gen. Genet.* 253:189–97
74. Fernández-Cañón JM, Peñalva MA. 1995. Overexpression of two penicillin structural genes in *Aspergillus nidulans*. *Mol. Gen. Genet.* 246:110–18
75. Veenstra AE, van Solingen P, Bovenberg RAL, van der Voort LHM. 1991. Strain improvement of *Penicillium chrysogenum* by recombinant DNA techniques. *J. Biotechnol.* 17:81–90
76. Malla S, Niraula NP, Liou K, Sohng JK. 2009. Enhancement of doxorubicin production by expression of structural sugar biosynthesis and glycosyltransferase genes in *Streptomyces peucetius*. *J. Biosci. Bioeng.* 108:92–98
77. Malla S, Niraula NP, Liou K, Sohng JK. 2010. Self-resistance mechanism in *Streptomyces peucetius*: overexpression of *drrA*, *drrB* and *drrC* for doxorubicin enhancement. *Microbiol. Res.* 165:259–67
78. Chen Y, Smanski M, Shen B. 2010. Improvement of secondary metabolite production in *Streptomyces* by manipulating pathway regulation. *Appl. Microbiol. Biotechnol.* 86:19–25

79. Chen Y, Wendt-Pienkowski E, Shen B. 2008. Identification and utility of FdmR1 as a *Streptomyces* antibiotic regulatory protein activator for fredericamycin production in *Streptomyces griseus* ATCC 49344 and heterologous hosts. *J. Bacteriol.* 190:5587–96
80. Lombo F, Brana AF, Mendez C, Salas JA. 1999. The mithramycin gene cluster of *Streptomyces argillaceus* contains a positive regulatory gene and two repeated DNA sequences that are located at both ends of the cluster. *J. Bacteriol.* 181:642–47
81. Sakai K, Kinoshita H, Shimizu T, Nihira T. 2008. Construction of a citrinin gene cluster expression system in heterologous *Aspergillus oryzae*. *J. Biosci. Bioeng.* 106:466–72
82. Bergmann S, Schumann J, Scherlach K, Lange C, Brakhage AA, et al. 2007. Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*. *Nat. Chem. Biol.* 3:213–17
83. Chiang YM, Szewczyk E, Davidson AD, Keller N, Oakley BR, et al. 2009. A gene cluster containing two fungal polyketide synthases encodes the biosynthetic pathway for a polyketide, asperfuranone, in *Aspergillus nidulans*. *J. Am. Chem. Soc.* 131:2965–70
84. Smanski MJ, Peterson RM, Rajski SR, Shen B. 2009. Engineered *Streptomyces platensis* strains that overproduce antibiotics platensimycin and platencin. *Antimicrob. Agents Chemother.* 53:1299–304
85. Alper H, Fischer C, Nevoigt E, Stephanopoulos G. 2005. Tuning genetic control through promoter engineering. *Proc. Natl. Acad. Sci. USA* 102:12678–83
86. Nevoigt E, Kohnke J, Fischer CR, Alper H, Stahl U, et al. 2006. Engineering of promoter replacement cassettes for fine-tuning of gene expression in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 72:5266–73
87. Pfleger BF, Pitera DJ, Smolke CD, Keasling JD. 2006. Combinatorial engineering of intergenic regions in operons tunes expression of multiple genes. *Nat. Biotechnol.* 24:1027–32
88. Salis HM, Mirsky EA, Voigt CA. 2009. Automated design of synthetic ribosome binding sites to control protein expression. *Nat. Biotechnol.* 27:946–50
89. Kizer L, Pitera DJ, Pfleger BF, Keasling JD. 2008. Application of functional genomics to pathway optimization for increased isoprenoid production. *Appl. Environ. Microbiol.* 74:3229–41
90. Fox RJ, Huisman GW. 2008. Enzyme optimization: moving from blind evolution to statistical exploration of sequence function space. *Trends Biotechnol.* 26:132–38
91. Wang C-w, Oh M-K, Liao JC. 2000. Directed evolution of metabolically engineered *Escherichia coli* for carotenoid production. *Biotechnol. Prog.* 16:922–26
92. Paradise EM, Kirby J, Chan R, Keasling JD. 2008. Redirection of flux through the FPP branch-point in *Saccharomyces cerevisiae* by down-regulating squalene synthase. *Biotechnol. Bioeng.* 100:371–78
93. Xie X, Wong WW, Tang Y. 2007. Improving simvastatin bioconversion in *Escherichia coli* by deletion of *bioH*. *Metab. Eng.* 9:379–86
94. Lomovskaya N, Otten SL, Doi-Katayama Y, Fonstein L, Liu X-C, et al. 1999. Doxorubicin overproduction in *Streptomyces peucetius*: cloning and characterization of the *dnrU* ketoreductase and *dnrV* genes and the *doxA* cytochrome P-450 hydroxylase gene. *J. Bacteriol.* 181:305–18
95. Chemler JA, Fowler ZL, McHugh KP, Koffas MAG. 2010. Improving NADPH availability for natural product biosynthesis in *Escherichia coli* by metabolic engineering. *Metab. Eng.* 12:96–104
96. Sung BH, Lee JH, Kim SC. 2009. *Escherichia coli* genome engineering and minimization for the construction of a bioengine. In *Systems Biology and Biotechnology of Escherichia coli*, ed. SY Lee, pp. 19–40. London: Springer Science + Business Media
97. Komatsu M, Uchiyama T, Omura S, Cane DE, Ikeda H. 2010. Genome-minimized *Streptomyces* host for the heterologous expression of secondary metabolism. *Proc. Natl. Acad. Sci. USA* 107:2646–51
98. Floss HG. 2006. Combinatorial biosynthesis—potential and problems. *J. Biotechnol.* 124:242–57
99. Baltz RH. 2006. Molecular engineering approaches to peptide, polyketide and other antibiotics. *Nat. Biotechnol.* 24:1533–40
100. Madduri K, Kennedy J, Rivola G, Inventi-Solari A, Filippini S, et al. 1998. Production of the antitumor drug epirubicin (4′-epidoxorubicin) and its precursor by a genetically engineered strain of *Streptomyces peucetius*. *Nat. Biotechnol.* 16:69–74
101. Fischbach MA, Walsh CT, Clardy J. 2008. The evolution of gene collectives: How natural selection drives chemical innovation. *Proc. Natl. Acad. Sci. USA* 105:4601–8

102. Mendes M, Recio E, Fouces R, Luiten R, Martin J, et al. 2001. Engineered biosynthesis of novel polyenes: a pimaricin derivative produced by targeted gene disruption in *Streptomyces natalensis*. *Chem. Biol.* 8:635–44
103. Remsing LL, Gonzalez AM, Nur-e-Alam M, Fernandez-Lozano MJ, Brana AF, et al. 2003. Mithramycin SK, a novel antitumor drug with improved therapeutic index, mithramycin SA, and demycarosyl-mithramycin SK: three new products generated in the mithramycin producer *Streptomyces argillaceus* through combinatorial biosynthesis. *J. Am. Chem. Soc.* 125:5745–53
104. Ito T, Roongsawang N, Shirasaka N, Lu W, Flatt PM, et al. 2009. Deciphering pactamycin biosynthesis and engineered production of new pactamycin analogues. *ChemBioChem* 10:2253–65
105. Ju J, Li W, Yuan Q, Peters NR, Hoffmann FM, et al. 2009. Functional characterization of *ttmM* unveils new tautomycin analogs and insight into tautomycin biosynthesis and activity. *Org. Lett.* 11:1639–42
106. Zhang M-Q, Gaisser S, Nur-E-Alam M, Sheehan LS, Vousden WA, et al. 2008. Optimizing natural products by biosynthetic engineering: discovery of nonquinone Hsp90 inhibitors. *J. Med. Chem.* 51:5494–97
107. Tang L, Chung L, Carney JR, Starks CM, Licari P, et al. 2005. Generation of new epothilones by genetic engineering of a polyketide synthase in *Myxococcus xanthus*. *J. Antibiot.* 58:178–84
108. Power P, Dunne T, Murphy B, Nic Lochlainn L, Rai D, et al. 2008. Engineered synthesis of 7-oxo- and 15-deoxy-15-oxo-amphotericins: insights into structure-activity relationships in polyene antibiotics. *Chem. Biol.* 15:78–86
109. Khaw L, Bohm G, Metcalfe S, Staunton J, Leadlay P. 1998. Mutational biosynthesis of novel rapamycins by a strain of *Streptomyces hygroscopicus* NRRL 5491 disrupted in *rapL*, encoding a putative lysine cyclodeaminase. *J. Bacteriol.* 180:809–14
110. Weist S, Bister B, Puk O, Bischoff D, Pelzer S, et al. 2002. Fluorobalhimycin—a new chapter in glycopeptide antibiotic research. *Angew. Chem. Int. Ed.* 41:3383–85
111. Galm U, Dessoy MA, Schmidt J, Wessjohann LA, Heide L. 2004. In vitro and in vivo production of new aminocoumarins by a combined biochemical, genetic, and synthetic approach. *Chem. Biol.* 11:173–83
112. Li SM, Heide L. 2005. New aminocoumarin antibiotics from genetically engineered *Streptomyces* strains. *Curr. Med. Chem.* 12:419–27
113. Kim W, Lee JS, Lee D, Cai XF, Shin JC, et al. 2007. Mutasynthesis of geldanamycin by the disruption of a gene producing starter unit: generation of structural diversity at the benzoquinone ring. *ChemBioChem* 8:1491–94
114. Menzella HG, Tran TT, Carney JR, Lau-Wee J, Galazzo J, et al. 2009. Potent non-benzoquinone ansamycin heat shock protein 90 inhibitors from genetic engineering of *Streptomyces hygroscopicus*. *J. Med. Chem.* 52:1518–21
115. Eustaquio AS, Moore BS. 2008. Mutasynthesis of fluorosalinosporamide, a potent and reversible inhibitor of the proteasome. *Angew. Chem. Int. Ed.* 47:3936–38
116. Eustáquio A, O'Hagan D, Moore B. 2010. Engineering fluorometabolite production: fluorinase expression in *Salinispora tropica* yields fluorosalinosporamide. *J. Nat. Prod.* 73:378–82
117. Runguphan W, Maresh JJ, O'Connor SE. 2009. Silencing of tryptamine biosynthesis for production of nonnatural alkaloids in plant culture. *Proc. Natl. Acad. Sci. USA* 106:13673–78
118. Weissman KJ. 2007. Mutasynthesis—uniting chemistry and genetics for drug discovery. *Trends Biotechnol.* 25:139–42
119. Hopwood DA, Malpartida F, Kieser HM, Ikeda H, Duncan J, et al. 1985. Production of "hybrid" antibiotics by genetic engineering. *Nature* 314:642–44
120. de Boer AL, Schmidt-Dannert C. 2003. Recent efforts in engineering microbial cells to produce new chemical compounds. *Curr. Opin. Chem. Biol.* 7:273–78
121. Challis GL. 2008. Genome mining for novel natural product discovery. *J. Med. Chem.* 51:2618–28
122. Pfeifer BA, Khosla C. 2001. Biosynthesis of polyketides in heterologous hosts. *Microbiol. Mol. Biol. Rev.* 65:106–18
123. Cane DE, Walsh CT, Khosla C. 1998. Harnessing the biosynthetic code: combinations, permutations, and mutations. *Science* 282:63–68
124. Moyne AL, Cleveland TE, Tuzun S. 2004. Molecular characterization and analysis of the operon encoding the antifungal lipopeptide bacillomycin D. *FEMS Microbiol. Lett.* 234:43–49

125. Reeves CD, Ward SL, Revill WP, Suzuki H, Marcus M, et al. 2004. Production of hybrid 16-membered macrolides by expressing combinations of polyketide synthase genes in engineered *Streptomyces fradiae* hosts. *Chem. Biol.* 11:1465–72
126. McDaniel R, Thamchaipenet A, Gustafsson C, Fu H, Betlach M, et al. 1999. Multiple genetic modifications of the erythromycin polyketide synthase to produce a library of novel "unnatural" natural products. *Proc. Natl. Acad. Sci. USA* 96:1846–51
127. Rascher A, Hu ZH, Viswanathan N, Schirmer A, Reid R, et al. 2003. Cloning and characterization of a gene cluster for geldanamycin production in *Streptomyces hygroscopicus* NRRL 3602. *FEMS Microbiol. Lett.* 218:223–30
128. Patel K, Piagentini M, Rascher A, Tian ZQ, Buchanan GO, et al. 2004. Engineered biosynthesis of geldanamycin analogs for Hsp90 inhibition. *Chem. Biol.* 11:1625–33
129. Gaisser S, Kellenberger L, Kaja AL, Weston AJ, Lill RE, et al. 2003. Direct production of ivermectin-like drugs after domain exchange in the avermectin polyketide synthase of *Streptomyces avermitilis* ATCC31272. *Org. Biomol. Chem.* 1:2840–47
130. Miao V, Coeffet-Le Gal MF, Nguyen K, Brian P, Penn J, et al. 2006. Genetic engineering in *Streptomyces roseosporus* to produce hybrid lipopeptide antibiotics. *Chem. Biol.* 13:269–76
131. Nguyen KT, Ritz D, Gu JQ, Alexander D, Chu M, et al. 2006. Combinatorial biosynthesis of novel antibiotics related to daptomycin. *Proc. Natl. Acad. Sci. USA* 103:17462–67
132. Coeffet-Le Gal MF, Thurston L, Rich P, Miao V, Baltz RH. 2006. Complementation of daptomycin *dptA* and *dptD* deletion mutations in trans and production of hybrid lipopeptide antibiotics. *Microbiology* 152:2993–3001
133. Nguyen KT, He XW, Alexander DC, Li C, Gu JQ, et al. 2010. Genetically engineered lipopeptide antibiotics related to A54145 and daptomycin with improved properties. *Antimicrob. Agents Chemother.* 54:1404–13
134. Rodriguez E, McDaniel R. 2001. Combinatorial biosynthesis of antimicrobials and other natural products. *Curr. Opin. Microbiol.* 4:526–34
135. Baltz RH. 2009. Daptomycin: mechanisms of action and resistance, and biosynthetic engineering. *Curr. Opin. Chem. Biol.* 13:144–51
136. Park SR, Han AR, Ban YH, Yoo YJ, Kim EJ, et al. 2010. Genetic engineering of macrolide biosynthesis: past advances, current state, and future prospects. *Appl. Environ. Microbiol.* 85:1227–39
137. Strieker M, Marahiel MA. 2009. The structural diversity of acidic lipopeptide antibiotics. *ChemBioChem* 10:607–16
138. Weissman KJ, Leadlay PF. 2005. Combinatorial biosynthesis of reduced polyketides. *Nat. Rev. Microbiol.* 3:925–36
139. Mendez C, Salas JA. 2001. Altering the glycosylation pattern of bioactive compounds. *Trends Biotechnol.* 19:449–56
140. Luzhetskyy A, Bechthold A. 2005. It works: combinatorial biosynthesis for generating novel glycosylated compounds. *Mol. Microbiol.* 58:3–5
141. Olano C, Mendez C, Salas JA. 2010. Post-PKS tailoring steps in natural product-producing actinomycetes from the perspective of combinatorial biosynthesis. *Nat. Prod. Rep.* 27:571–616
142. Flatman RH, Eustaquio A, Li SM, Heide L, Maxwell A. 2006. Structure-activity relationships of aminocoumarin-type gyrase and topoisomerase IV inhibitors obtained by combinatorial biosynthesis. *Antimicrob. Agents Chemother.* 50:1136–42
143. Sanchez C, Zhu LL, Brana AF, Salas AP, Rohr J, et al. 2005. Combinatorial biosynthesis of antitumor indolocarbazole compounds. *Proc. Natl. Acad. Sci. USA* 102:461–66
144. Sanchez C, Salas AP, Brana AF, Palomino M, Pineda-Lucena A, et al. 2009. Generation of potent and selective kinase inhibitors by combinatorial biosynthesis of glycosylated indolocarbazoles. *Chem. Commun.* 2009:4118–20
145. Werneburg M, Busch B, He J, Richter ME, Xiang L, et al. 2010. Exploiting enzymatic promiscuity to engineer a focused library of highly selective antifungal and antiproliferative aureothin analogues. *J. Am. Chem. Soc.* 132:10407–13
146. Horinouchi S. 2008. Combinatorial biosynthesis of non-bacterial and unnatural flavonoids, stilbenoids and curcuminoids by microorganisms. *J. Antibiot.* 61:709–28

147. Katsuyama Y, Funa N, Miyahisa I, Horinouchi S. 2007. Synthesis of unnatural flavonoids and stilbenes by exploiting the plant biosynthetic pathway in *Escherichia coli*. *Chem. Biol.* 14:613–21
148. Leonard E, Runguphan W, O'Connor S, Prather KJ. 2009. Opportunities in metabolic engineering to facilitate scalable alkaloid production. *Nat. Chem. Biol.* 5:292–300
149. Runguphan W, Qu X, O'Connor SE. 2010. Integrating carbon-halogen bond formation into medicinal plant metabolism. *Nature* 468:461–64.
150. Williams GJ, Goff RD, Zhang C, Thorson JS. 2008. Optimizing glycosyltransferase specificity via "hot spot" saturation mutagenesis presents a catalyst for novobiocin glycorandomization. *Chem. Biol.* 15:393–401
151. Williams GJ, Zhang C, Thorson JS. 2007. Expanding the promiscuity of a natural-product glycosyltransferase by directed evolution. *Nat. Chem. Biol.* 3:657–62
152. Chen S, Galan MC, Coltharp C, O'Connor SE. 2006. Redesign of a central enzyme in alkaloid biosynthesis. *Chem. Biol.* 13:1137–41
153. Runguphan W, O'Connor SE. 2009. Metabolic reprogramming of periwinkle plant culture. *Nat. Chem. Biol.* 5:151–53
154. Schmidt-Dannert C, Umeno D, Arnold FH. 2000. Molecular breeding of carotenoid biosynthetic pathways. *Nat. Biotechnol.* 18:750–53
155. Yoshikuni Y, Ferrin TE, Keasling JD. 2006. Designed divergent evolution of enzyme function. *Nature* 440:1078–82
156. Hans M, Hornung A, Dziarnowski A, Cane DE, Khosla C. 2003. Mechanistic analysis of acyl transferase domain exchange in polyketide synthase modules. *J. Am. Chem. Soc.* 125:5366–74
157. Hunziker D, Yu TW, Hutchinson CR, Floss HG, Khosla C. 1998. Primer unit specificity in rifamycin biosynthesis principally resides in the later stages of the biosynthetic pathways. *J. Am. Chem. Soc.* 120:1092–93
158. Zhou Z, Lai JR, Walsh CT. 2007. Directed evolution of aryl carrier proteins in the enterobactin synthetase. *Proc. Natl. Acad. Sci. USA* 104:11621–26
159. Fischbach MA, Lai JR, Roche ED, Walsh CT, Liu DR. 2007. Directed evolution can rapidly improve the activity of chimeric assembly-line enzymes. *Proc. Natl. Acad. Sci. USA* 104:11951–56

Annual Review of Chemical and Biomolecular Engineering

Volume 2, 2011

Contents