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Metabolic Engineering for the Production of Natural Products

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Abstract

Natural products and natural product derived compounds 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 and pharmaceutically 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 have not only yielded fascinating scientific discoveries but also make it an increasingly viable approach for the optimization of natural product biosynthesis.

Keywords

Strain Improvement; Secondary Metabolism; Combinatorial Biosynthesis

INTRODUCTION

Natural products produced by plants, bacteria, and fungi have been a rich source of bioactive compounds for drug discovery and development (Figure 1). Natural products dominated early drug discovery as large screening programs were set up following the breakthrough isolation and medicinal application of penicillin in the 1940s (1). As of 1990 80% of drugs in use were natural products or natural product inspired (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 the period 1981–2006, 52% of the new chemical entities approved by the FDA were natural products or natural product inspired (3). Natural products are also termed secondary metabolites, or those not required for growth of the producing organisms. Unlike primary metabolites which are required for growth and are

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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. Many of these compounds are structurally complex, containing multiple chiral centers and labile connectivities, which 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 (4).

Natural product discovery and process development are very 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 (5), has gained increasing interest as a way to develop high titer bioprocesses and produce new “non-natural” natural products. Advances in molecular biology techniques and knowledge of metabolic pathways have enabled unprecedented amount of control over these complex biosynthetic processes. The dramatic decreases in the cost of sequencing technology has resulted in an exponential amount of genomic data available 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 avoiding unintended consequences which may not be clear by local pathway analysis alone (6). Furthermore, advances in protein engineering have led not only to both improved enzyme efficiency, but also custom-tailored enzyme functions (7). Using these tools and more, metabolic engineering has been applied towards two main objectives in natural product biosynthesis: 1) to increase the titer of the target compound, and 2) to modify the natural product scaffold for improved pharmacological properties. Although many of the examples are compound-specific, we will discuss some general strategies used towards achieving 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 (8). 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 what is necessary for industrial scale production (9). Additionally, there has been an increasing number of so called “cryptic pathways” discovered which do not actively produce any metabolites. These pathways can be activated under certain conditions, many of which are unknown. Mining the metabolites in these pathways therefore represents a new source of obtaining new natural products (10). These factors necessitate that producing organisms undergo significant strain improvement in order to produce the desired metabolite at an industrial scale.

Traditionally, strain improvement was achieved by screening for the highest native producing strain and further improving the strain by rounds of mutation and selection (9). 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 based on this “black box” approach further improved production; and industrial strains are now estimated to produce penicillin at 100,000-fold higher than the original strain (11). 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 rate of isolating a mutant with improved properties is estimated to be on the order of 1 in 10,000 (9). With advances in our understanding of metabolic pathways and improving genetic tools, rational strain improvement by metabolic engineering is a promising tool for improving natural product yield. There are many genetic strategies that 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 byproducts 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 will cover methods employed for metabolic engineering of natural product pathways for improved efficiency and yield, with a focus on biosynthesis in microorganisms.

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 is subjected to 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 the natural product antibiotics produced by microorganisms. Of these, over 75% are produced by actinomycetes which includes the prolific *Streptomyces* genus (12). The largest groups of natural products produced by bacteria are polyketides, such as erythromycin and tetracycline, nonribosomal peptides (NRPs) such as daptomycin and vancomycin, hybrid polyketide/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 (13). However, the growth time of actinomycetes is still slow compared to 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 bacterial derived natural products are either heterologous *Streptomyces* hosts or the well-characterized *Escherichia coli*. *Streptomyces* hosts are advantageous as many already produce antibiotics and therefore have the pathways for necessary precursor molecules. Additionally *Streptomyces* bacteria are physiologically very different from *E. coli* and some proteins from *Streptomyces* pathways have been unable to be successfully expressed in *E. coli*. An example is the KS-CLF heterodimer which is

responsible for synthesis of the poly- β -ketone backbone during bacterial aromatic polyketide synthesis and has so far not been successfully expressed in *E. coli*. *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 interfere with the transplanted heterologous pathway. Several engineered strains have been constructed including *S. coelicolor* CH999 (14), and *S. lividans* K4–114 (15) which have their native antibiotic pathways knocked out to create a clean background for production of a new compound. For example, heterologous production and ease of purification of NRP daptomycin in *S. lividans* was increased upon elimination of the actinorhodin producing pathway (16). Another strategy is to select an industrial strain that has already been optimized through mutation and introduce a pathway of interest. Recently Reynolds and coworkers utilized an industrial monesin producer, *Streptomyces cinnamonensis*, to produce the polyketide antibiotic tetracenomycin in titers significantly higher than the native producer (17). Many of the mutations 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, *E. coli* still offers a much shorter growth time and easier genetic manipulations than actinomycetes hosts and many bacterial natural product pathways have been successfully expressed in *E. coli*, including type I modular polyketide synthases (PKSs) (18–20), nonribosomal peptide synthetases (NRPSs) (21, 22), and hybrid PKS/NRPS systems (23, 24). The first successful example of heterologous expression of a complex polyketide pathway in *E. coli* was erythromycin (18). Erythromycin is produced by a type I modular PKS consisting of three multi-domain modules DEBS 1 (370 kDa), DEBS 2 (380 kDa) and DEBS 3 (332 kDa) which together produce the macrolide core 6-deoxyerythronolide B (6dEB). One consideration when using *E. coli* as a host is that not all of the necessary biosynthetic components or substrates may be available. For polyketide biosynthesis, the PKS must be posttranslationally modified by a phosphopantetheine transferase (PPTase). To achieve this, the authors 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. Additional modifications to increase 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 6dEB at a rate of 0.1 mmol per gram of cellular protein per day (18). 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 6dEB 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 the native producer (20). Like PKSs, NRPSs also require posttranslational pantetheinylation of the individual thiolation domains to be fully functional. Watanabe and coworkers achieved total biosynthesis of antitumor NRP

echinomycin by introducing a three plasmid system carrying the echinomycin biosynthetic genes from *Streptomyces lasaliensis*, *sfp*, *fabC* encoding fatty acid acyl carrier protein, and a gene conferring echinomycin resistance into *E. coli* (22). 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. Additional reviews can be found which elaborate further on heterologous biosynthesis of bacterial natural products (25–28).

Natural Products from Fungi—Fungi are responsible for approximately 30% of microbial natural products, a share which is on the rise due to increasing interest in fungal natural products (12). Major classes of fungal natural products are polyketides, peptide based compounds such as NRPs and β -lactams, terpenoids and combinations of these (29). 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 extent of bacterial natural products (29). Many fungi are already good hosts for secondary metabolite production, as they grow well on cheap carbon sources, and have had great success with traditional strain improvement and process improvement strategies resulting in multi-gram per liter yields of the desired pharmaceutical products (30).

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 PCR that is required to ensure correct expression in *E. coli* and yeast (31). Like the actinomycetes, several of the more robust, well characterized fungal strains may be good candidates for heterologous hosts for gene clusters which originate from more recalcitrant strains. The first example of a metabolite produced by a fungal synthase in a heterologous host is 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 (32), *E. coli* (33), *Saccharomyces cerevisiae* (33), and *Aspergillus nidulans* (34). In all but the fungal host, the introns were removed from the gene encoding 6-MSAS. Without any further optimization, yields from bacteria were around 60–75 mg/L (32, 33), while production in *A. nidulans* was over 300 mg/L (34) and yeast produced 1.7 g/L (twice that of native producer *Penicillium patulum*) (33). More recent examples in *E. coli* and fungal hosts include the heterologous production of beauvericin in *E. coli* (35) and reconstruction of the four gene pathway to produce tenellin in *Aspergillus oryzae* (36). Heterologous hosts have also been used to express fungal synthases for in vitro study. The highly reducing iterative PKS LovB from the lovastatin pathway was recently expressed and purified from an engineered *S. cerevisiae* strain (37). Though 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 (31, 38, 39).

Natural Products from Plants—Plants are also prolific producers of pharmaceutically important metabolites including terpenoids such as artemisinin and paclitaxel, alkaloids such as camptothecin, and polyketides 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 be able to produce these valuable compounds in microbial hosts. The higher complexity of plants, however also 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 successfully produced in both *E. coli* and yeast including precursors to the medicinally important terpenoids paclitaxel (40, 41) and artemisinin (42, 43), and alkaloid intermediate reticuline (44, 45).

The plant aromatic polyketides, such as the flavonoids and stilbenes, have also been successfully produced in *E. coli* host using an artificial pathway construction approach, in which genes from different pathways in different plants were combined to reconstruct a functional pathway (46). For example, for the biosynthesis of flavanone naringenin in *E. coli*, the authors assembled a pathway consisting of a phenylalanine ammonia-lyase (PAL) from the yeast *Rhodotorula rubra*, a cinnamate/coumarate:CoA Ligase (ScCCL) from *S. coelicolor*, a chalcone synthase (CHS) from the licorice plant *Glycyrrhiza echinata* and chalcone isomerase (CHI) from *Pueraria lobata*. This hybrid pathway produced naringenin at 57 mg/L (47).

Additionally, some progress has been made to address the problem of the plant derived cytochrome P450 expression (48). Several groups have had success in improving P450 expression in *E. coli*, using strategies such as modifying the *N*-terminal membrane recognition domain (49, 50). Once the target compound can be produced, one can take advantage of the range of tools and models available for well characterized hosts like *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 of which it can be engineered for increased yield.

Increasing Precursor Supply

Increasing precursor supply has been successfully used for most of the major classes of natural products and can be applied both to 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 primary metabolites. Malonyl-CoA is an important building block for the biosynthesis of polyketides, and is produced by carboxylation of acetyl-CoA by the acetyl-CoA carboxylase complex (ACCase). Ryu and coworkers engineered *S. coelicolor* for increased production of malonyl-CoA by overexpressing the genes encoding ACCase. The result was a 6-fold increase in actinorhodin production (51). In addition to overexpression of ACCase, Zha and coworkers combined several strategies to increase malonyl-CoA levels in *E. coli* such as overexpression of gene encoding acetyl-CoA synthetases, knockout of competing pathways and elimination of

malonyl-CoA degrading pathways for a 15-fold increase in intracellular malonyl-CoA (52). Fowler et. al. utilized a model based approach to identify and verify new genetic manipulations to increase flux to malonyl-CoA resulting in enhanced production of flavonoids in *E. coli* (53). In addition to malonyl-CoA, type I polyketides 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 supply of these building blocks for an increase in desired polyketide yield (54–56).

Terpenoid producing pathways utilize isopentenyl pyrophosphate (IPP) as a building block to generate the longer terpenoid backbones of geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) precursors to terpenoid natural products. IPP is produced predominantly by the 2C-methyl-D-erythritol-4-phosphate (MEP) or nonmevalonate pathway in prokaryotes and the chloroplasts of higher plants, and the mevalonate (MVA) pathway in eukaryotes and the cytosol of higher plants (57). Precursor engineering strategies in *E. coli* have either focused on 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 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 (58–60). Martin and coworkers bypassed the native pathway, and instead introduced the MVA pathway from *S. cerevisiae* to *E. coli*, resulting in greatly increased production of isoprenoid precursors (42). Further optimization by balancing individual enzyme expression relieved growth inhibition caused by accumulation of toxic 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) intermediate, and resulted in further increase of isoprenoid production (61). In yeast, the flux through the native MVA pathway was increased by chromosomal integration of engineered form of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), a bottleneck in the MVA pathway, resulting in a five-fold increase in amorphaadiene production (43). 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 synthetases, thereby increasing flux from pyruvate to acetyl-CoA (62).

The shikimate pathway is a vital part of the primary metabolism, providing 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 nonribosomal peptides (63). Increasing flux through the shikimate pathway or downstream amino acid tailoring steps has been used successfully to increase natural product yields. Balhimycin is a glycopeptide antibiotic which shares the same 7-amino acid backbone as the pharmaceutically important vancomycin, differing only in the glycosylation patterns. 5 of the 7 amino acids that make up the aglycon are either directly or indirectly derived from the shikimate pathway. By overexpressing 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, the first enzyme in the shikimate pathway, balhimycin specific productivity was increased by 250% (64).

Engineering Natural Product Pathways

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 (65–67). 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, resulting in up to 64-fold increase in penicillin production (66). While this may be the simplest way for nature to evolve gene dosing strategies, this strategy may be somewhat wasteful as it increases expression of all the biosynthetic enzymes rather than targeting bottlenecks in the pathway. Metabolic engineering allows one to fine tune the expression of individual enzymes by gene dosing (68), transcriptional regulation (69), or protein engineering (70) approaches. For example during the biosynthesis of penicillin in *A. nidulans*, it was found that aminoadipyl-cysteiny-valine synthetase (ACVS) was the bottleneck enzyme and overexpression of the gene encoding ACVS resulted in 100-fold increase in ACVS expression and a corresponding 30-fold increase in penicillin (71). Overexpression of genes encoding downstream tailoring enzymes isopenicillin N synthetase (IPNS) and acyltransferase (ACYT), however resulted in only a slight increase in penicillin (72). Interestingly, the opposite effect was seen for *P. chrysogenum* where an acyltransferase encoded by *penDE* was shown to be the rate limiting enzyme (73). During the biosynthesis of anticancer aromatic polyketide doxorubicin, glycosylation was determined to be the rate limiting step. By overexpressing genes responsible for deoxysugar biosynthesis and glycosyltransfer, Malla and coworkers observed a nearly 6-fold increase in doxorubicin production (74). Another important consideration especially with the production of bioactive natural products, is that production levels may not only be limited 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 with doxorubicin biosynthesis, overexpression of resistance genes was required to obtain further improvements in product yield to alleviate product inhibition of late stage tailoring enzyme DoxA, and toxic effects of doxorubicin (75).

Manipulating the Regulatory Component of a Pathway

In many organisms, pathways specific regulators may be utilized to enhance production of the resulting natural product. Many gene clusters identified in *Streptomyces* encode a *Streptomyces* antibiotic regulatory protein (SARP) which has been shown to be a positive regulator of antibiotic production (76). Overexpression of the SARP from the fredericamycin producing gene cluster of *Streptomyces griseus* ATCC 49344 resulted in a nearly 6-fold increase in antibiotic production over the native strain (77). Overexpression of SARP MtmR from the mithramycin gene cluster in *Streptomyces argillaceus* resulted in a 16-fold increase of mithramycin titer. Interestingly, in the same study authors found that MtmR was also able to activate the actinorhodin producing pathway when heterologously expressed in *S. coelicolor* (78). Using 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* (79). This also indicates the importance of the native regulator when transferring an entire gene cluster which retains the original promoters and control elements from one organism to another. This strategy has also been used to turn on cryptic pathways in fungi (10), and has led to the identification of two novel metabolites, aspyridone and asperfuranone, from *A. nidulans* (80, 81).

Regulation of secondary metabolite pathways can also include negative regulation by pathway specific repressors. The gene cluster responsible for the biosynthesis of 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 improved the titer of platensimycin and platencin by 100-fold over the wild type strain (82).

Tunable Control of Metabolic Pathway Components

When assembling a metabolic pathway from the ground up (gene by gene), there is additional opportunity 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* (83) and yeast (84) 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. Pflieger and coworkers developed a library of tunable intergenic regions (TIGRs) that control expression levels by differences in mRNA secondary structures, RNase cleavage sites and RBS sequestering sequences (85). Recently, the Voigt group developed a method for predictable control of protein expression based on design of RBS sequences (86). As mentioned earlier, finding the proper balance of enzyme levels was key to reducing toxicity observed during amorphaadiene production in *E. coli* (61). Determining the proper enzyme levels, however, is an additional challenge. In an example by Hawkins & Smolke for the heterologous production of benzylisoquinoline alkaloids (BIAs) in *S. cerevisiae*, the authors first utilized an inducible promoter system to titrate gene expression one by one and determine optimal expression levels for each gene (45). These optimal expression levels were then correlated to expression levels obtained by members of the library of mutant TEF1 promoters developed by Nevoigt et. al (84) to construct a new strain with engineered promoters chosen to reflect the optimized enzyme expression levels (45). 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. Kizer and coworkers utilized a transcriptomics and metabolomics approach to identify and ameliorate an imbalance in carbon flux during isoprenoid biosynthesis (87).

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 due to the poor expression and activity of the native enzymes

in *E. coli* and other heterologous hosts, resulting in increased production of the tailored final product. Leonard and coworkers 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 P450BM-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 (49). Chang et. al. successfully expressed a terpenoid P450 hydroxylase in *E. coli* by modification of the N-terminal membrane anchor and optimization of the CPR redox partner (50). In another case of rational engineering, homology guided point mutation was used to alter the active site of levopimaradiene synthase for improved productivity (70). Directed evolution is also a powerful tool to improve enzyme activity (88). 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 (70, 89).

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, endogenous yeast squalene synthase encoded by *erg9* also utilizes the sesquiterpene precursor farnesyl-pyrophosphate (FPP) used by amorphaadiene synthase. Thus knocking out the competing *erg9* gene resulted in an increase in amorphaadiene synthesis (90). During in vivo bioconversion of lovastatin intermediate monacolin J to simvastatin using *E. coli* expressing heterologous acyltransferase LovD, it was found that *E. coli* could unexpectedly hydrolyze the synthetic thioester substrate (91). The responsible hydrolase BioH was then knocked out to improve simvastatin production (91). During doxorubicin biosynthesis, several genes encoded by the *dxr* gene cluster were deleted to improve titer as the corresponding protein products acted on doxorubicin to convert it to a less active derivatives (92).

In addition to obvious targets which 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 which may place a metabolic burden on the cell as flux through the pathway is increased. Chemler et. al. 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 2–4 fold (93). Due to the involvement of NADP(H) in a large number of cellular enzymatic reactions, the effective solution space would be impossible to probe experimentally, making 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 “non-essential” 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 resulting in strains with genome size up to 22% reduced from wild-type with no growth deficiency (30% reduction achieved with growth deficiency) and improved genome stability and production of target metabolites (94). A genome minimized *Streptomyces* host has recently been reported by Komatsu and coworkers. 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* (95). These reports show promise that genome minimization may be a feasible tactic to streamline biochemical production, however 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. In addition to acting on antimicrobial targets, the homology in many protein structural folds and receptors across different organisms has allowed the exploitation of these small molecules to act on drug targets that are irrelevant to the producing organisms (96). However, these natural products are often not optimized for human drug targets and may have undesirable properties or side effects clinically (eg. toxicity, low oral availability etc.). Therefore, many natural product-based drugs have minor structural modifications from the original natural product scaffold, which 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, where 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 an increasing interest in the application of metabolic engineering as a complementary tool for this purpose (97). In cases where the natural products are highly complex, metabolic engineering may allow selective modifications of natural product scaffolds, which may be difficult to achieve by chemical means alone. Another avenue for engineering natural product biosynthesis is to 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*, eliminating the need for post-fermentation processing through the low-yielding semisynthetic route (98). By integrating the downstream synthetic steps into biological systems, it is possible to produce those 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 1) the deletion or recruitment of new genes in the biosynthetic pathway or 2) mutations and recombination in individual genes, which resulted in altered activity of the enzymes (99). Metabolic engineering essentially allows us to accelerate and control the evolution processes to select for natural product analogs with useful biological activities. We will focus here on the common metabolic engineering strategies useful for exploring the chemical space around the 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 via 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 pimarin gene cluster (100). Disruption of a ketoreductase gene in mithramycin pathway led to isolation of three analogs, including mithramycin SK with an improved therapeutic index compared to the parent drug (101). Two recent examples illustrate the application of targeted gene disruption to generate derivatives of pactamycin (102) and tautomycin (103), which 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 (104). The novel nonquinone macbecin analog has almost 100-fold improvement in Hsp90 binding affinity and significantly reduced toxicity.

Beside deletion of whole genes, structural variation could also be introduced by inactivation of individual domain within the multidomain modular megasynthases like PKSs and NRPSs. Ketoreductase (KR) domain inactivation of PKSs had been used to generate analogs of epothilones, potent cytotoxic agents with a semisynthetic derivative (ixabepilone) recently approved as a chemotherapeutic drug (105). 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 (106). 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 disrupting the gene that produces the natural substrate, a substitute (so called ‘mutasynthon’) could be fed into the growth medium to generate new analogs. This has been successfully applied in generation of new analogs for broad classes of compounds, such as the rapamycins (macrolides) (107), balhimycin (glycopeptides) (108) and novobiocin/chlorobiocin (aminocoumarins) (109, 110), illustrating the versatility of this strategy. Recently, this approach was used to generate nonbenzoquinone analogs of the Hsp90 inhibitor geldanamycin (111, 112). By removing the biosynthetic genes for the starter unit 3-amino-5-hydroxybenzoic acid (AHBA) 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 (112).

Another good example is the mutasynthesis of fluorosalinoporamide by disruption of the S-adenosylmethionine (SAM)-dependent chlorinase (*salL*) and feeding with 5'-fluoro-5'-deoxyadenosine (5'-FDA) in the salinoporamide-producing *Salinispora tropica* (113). The resultant fluoro-substituted compound has reversible proteasome inhibitory activity, which is unlike its chloro-substituted parent salinoporamide. A fluorinase *flA* from *Streptomyces cattleya* was later inserted into the producing host to bypass 5'-FDA feeding (114).

Recently, O'Connor and co-workers demonstrated that mutasynthesis can also be employed in plants by gene-silencing methods. RNA mediated suppression of tryptamine biosynthesis in *Catharanthus roseus* followed by feeding with 5-fluorotryptamine yielded several fluoro-substituted alkaloids (115). A short perspective on the application of mutasynthesis in drug discovery and development is available (116).

Pathway Engineering and Combinatorial Biosynthesis

In microorganisms, the most common modes of chemical diversification are horizontal transfer of whole or partial gene clusters following 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, and some that consist of more than one core structures. The aromatic polyketides, the acidic lipopeptides daptomycin/A54145, the macrolides, aminocourmarins and the teicoplanin families of antibiotics are some of the examples. The homologies and divergences observed among the genes encoding the pathways have given scientists the inspiration to reshuffle the genes and modules between closely related natural product pathways to create new combinations. In 1985, Hopwood and coworkers first demonstrated the feasibility of combinatorial biosynthesis using the pathway genes of three structurally related polyketides. By introducing genes from the actinorhodin pathway in *S. coelicolor* into medermycin- and dihydrogranaticin-producing *Streptomyces* species, new hybrid compounds, mederrhodin and dihydrogranatihordin, can be produced (117).

A pathway can often be manipulated by changing the combination of genes (or part of the genes) involved in the biosynthesis to yield new product analogs, providing that the enzymes at the downstream of the alteration point have sufficient promiscuity to tolerate the changes in the substrate (118). This implies that introducing a change at the later steps of a pathway often has higher success rate since fewer downstream enzymes are involved. Minor alteration at early points in the pathway can sometimes be tolerated by downstream enzymes as long as it does not disrupt the overall core scaffold. Since the biosynthetic enzymes for a family of related molecules act on similar substrates or intermediates, the chances for successfully producing chimeric molecules by swapping the genes within a family are considerably higher. This highlights the importance for continuous discovery and elucidation of new natural product pathways, and genome mining has emerged as a potential tool for such application. The engineering of the metabolic pathways 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

heterologous host is preferred when the native host is slow-growing or cannot be easily manipulated genetically (97, 119). The biosynthetic pathways can be first assembled or modified in a cloning host, such as in *E. coli*, follow by introduction of the whole engineered pathway into an appropriate heterologous host.

Engineering Modular Megasyntases—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, both at the module and domain level (120). A good example is the iturin family of nonribosomal peptides. From the structure of iturin A, bacillomycin D and mycosubtilin, and their corresponding NRPS genes, it is evident that the variation in the lower half of these molecules is the result of mutation and intergenic rearrangement of adenylation (A) domains in the NRPSs (121). The three 16-membered macrolides (tylosin, spiramycin and chalcomycin), also appear to be diverged from a common ancestral pathway as their corresponding gene clusters share five similar PKS subunits (122). On the same token, these modular megasyntases 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 megasyntases at the subunit, module and domain level (Figure 3A and B). The most studied groups of molecules are the macrolides and the lipopeptide antibiotics, which will be used as the main examples here.

In a landmark success about a decade ago, the group at Kosan Biosciences generated over 50 analogs of 6-deoxyerythronolide B (6-dEB), by swapping the acyltransferases (ATs) and β -carbon processing domains between 6-dEB and rapamycin PKS in an engineered heterologous host (Figure 3A) (123). With the discovery of the geldanamycin gene cluster (a macrolactam) in *Streptomyces hygroscopicus* (124), the researchers at Kosan Biosciences made a series of AT domain swapping with rapamycin PKSs to generate a number of derivatives, including one analog with a four-fold enhanced affinity for Hsp90 (125). Another significant example is the direct production of a semisynthetic drug, ivermectin, in a recombinant *S. avermitilis* strain harboring engineered PKSs. By substituting the DH and KR domains of module 2 of the avermectin PKS with the DH, ER and KR domains of module 13 from the rapamycin PKSs, 22,23-dihydroavermectins (ivermectin) were successfully produced (126), provided a route for producing the drug using fermentation alone. Besides exchanging the PKS domains, novel analogs can also be obtained by exchanging the whole PKS subunits. By shuffling the PKS subunits from three related 16-membered macrolide pathways mentioned above (tylosin, spiramycin and chalcomycin), hybrid molecules which varied in methyl, ethyl or methoxy side chains at several positions were produced (122).

The combinatorial biosynthesis effort to produce analogs of daptomycin, an antibiotic approved in the United States for treatment of skin and skin structure infections, was spearheaded by a group at Cubist Pharmaceuticals. The first successful production of hybrid lipopeptides was achieved by exchanging the third NRPS subunits between the structurally related daptomycin, A54145 and calcium-dependent antibiotic (127). Coupling of whole-subunit exchange with the deletion of the Glu12-methyltransferase gene, module exchange

and natural side chain variation, more than 70 new lipopeptides have been generated (Figure 3B), most of which have antibacterial activities (127–129). Daptomycin and many of the analogs have poor efficacy in the treatment of community-acquired pneumonia, likely due to sequestration in pulmonary surfactant. The latest extension of the work involved the generation of more analogs related to A54145 and daptomycin using similar combinatorial strategy to screen for improved antibacterial activity in the presence of bovine surfactant (130). 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, demonstrating the potential application of this approach in drug development.

It is useful to note that in all 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 generate further structural diversity. For example, by combining the 6-dEB synthase domain-swapping and variation in post-PKS tailoring, additional erythromycin and erythrolide analogs have been generated (131), illustrating 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 (97, 131–135).

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 (136). Combinatorial engineering of the glycosyltransferases from different pathways has allowed the generation of arrays of analogs differing in the glycosylation patterns for several classes of natural products, such as the glycopeptides related to vancomycin and teicoplanin, macrolides related to erythromycin and tylosin, indolcarbazoles related to rebeccamycin, and the aromatic polyketide anthracyclines and angucyclines (136, 137). Polyketides 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. (138)

The enzymatic toolbox available for modification of a common structural scaffold usually consists of genes encoding for the pathways of structural related compounds. For example, the aminocoumarin 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 novobiocin pathway with the chlorinase in clorobiocin pathway and vice versa, plus inactivation of the acyltransferase in clorobiocin pathway, Heidi and coworkers could produce a series of new hybrid aminocoumarins (110)

(Figure 3C), which provided valuable insights into the SAR of this class of gyrase and topoisomerase IV inhibitors (139). Similarly, equipped with the genetic blueprints of two structurally related indolecarbazoles (staurosporine and rebeccamycin) and tryptophan halogenases with different regioselectivity, Salas and co-workers were able to generate 32 rebeccamycin analogs by altering the patterns of glycosylation, methylation and halogenations, in a heterologous *Streptomyces albus* host (140). Recent extension of the work has led to the identification of two hybrid indolecarbazoles, which are potent and selective inhibitors of JAK2 and Ikkb kinases (141), underlying the potential of this strategy. More recently, Hertweck and co-workers 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 (142).

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 to microorganisms where the pathway genes tend to cluster together. This limits the toolbox for metabolic engineering and combinatorial biosynthesis. Using the artificial pathway approach with genes from different organisms, some success in combinatorial biosynthesis of plant metabolites have been achieved in the type III PKS pathways. Horinouchi and his co-workers divided the flavanoid biosynthetic pathway into three components: substrate synthesis, polyketide synthesis, and post-PKS modification. By varying these three components in separate plasmid systems, and coupled with precursor-directed biosynthesis, an array of novel and known plant polyketides were generated (46, 143, 144). Significantly, several of the novel stilbenes showed inhibition against the ethoxyresorufin-*O*-deethylase activity of CYP1B1 (144). Similar combinatorial approaches can be employed with the development of artificial pathway system for other plant metabolites, especially the alkaloids (145).

Protein Engineering

Despite the successful examples above, researchers are often confronted by the problem where the enzymes/domains downstream in the pathway have limited tolerance to the new substrate introduced by metabolic engineering. Protein engineering either by rational design or directed evolution has been a useful tool for improving the efficiency and stability of the enzymes for biocatalysis development and metabolic engineering for titer improvement. For natural product diversification, they present as attractive tools for increasing the substrate promiscuity or changing the substrate specificity of biosynthetic enzymes. For example, directed evolution has been used by Thorson and co-workers to expand the substrate promiscuity of glycosyltransferases to accept alternate aglycon and sugar substrates (146, 147). In another instance, O'Connor and co-workers 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 (148). The engineered strictosidine synthase was transformed into *C. roseus*, resulting in transgenic plant cell culture that can produce a variety of unnatural alkaloid compounds with precursor feeding, demonstrating the utility of enzyme engineering in mutasynthesis (149).

For directed evolution, a large, high-quality library, and an efficient screening strategy are essential. Schmidt-Dannert et al. exploited the optical properties of colored carotenoids, which allow screening of large number of enzyme variants visually (150). Directed evolution of the phytoene desaturase, which synthesizes the conjugated double bonds, led to isolation of a pink variant producing the fully conjugated carotenoid tetrahydrocyclopentadiene. 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 (150). Yoshikuni et al. also showed that it is possible to engineer a promiscuous biosynthetic enzyme to produce specific products (151). 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, where a missing enzyme in a pathway can be “borrowed” from a closely related promiscuous enzyme.

For modular PKS and NRPS megasynthases, a problem exists where simple swapping of functional domains often result in nonfunctional or heavily impaired chimerical enzymes (128, 152). The problem can be twofold: 1) 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 (153), and 2) the domain swapping disrupts the quaternary interactions between protein domains as demonstrated in a mechanistic study for AT domain swapping (152). Using the NRPS of the siderophore enterobactin as a model, Zhou et al. 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 non-cognate aryl-carrier protein (ArCP) domain (154). Fischbach et al. further demonstrated that limited rounds of directed evolution can improve the activity of a chimerical NRPS swapped with a heterologous A domain of the same substrate specificity as wild type, as well as a chimerical NRPS with a heterologous A domain that activates a different substrate (155).

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

PERSPECTIVE AND CONCLUDING REMARKS

Accessibility is one of the main hurdles in natural product drug discovery since many natural products are naturally produced in low yields in the native organisms, and sometimes

in multiple forms further complicating the identification, isolation, and structural elucidation. Furthermore, lead optimization with natural product scaffolds by chemical modification is usually more difficult and laborious as they often possess a variety of functional groups, which requires multiple protection and deprotection steps. Despite the large structural diversity and high hit rates, these undesirable properties have made natural products less attractive, and many pharmaceutical companies in the past had turned to combinatorial synthetic libraries as 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 at 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, and 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 only be obtained 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.

Though 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 titer achieved for industrial microorganisms, metabolic engineering can be a complementary approach to reduce the time needed to optimize a strain, simplify the 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 all the rich biochemical diversity that nature has to offer.

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ACRONYMS

PK	Polyketide
PKS	Polyketide synthase
NRP	Nonribosomal peptide
NRPS	Nonribosomal peptide synthase
PPTase	phosphopantetheine transferase
KS	Ketosynthase

AT	Acyl transferase
DH	Dehydratase
KR	Ketoreductase
ER	Enoyl reductase
ACP	Acyl carrier protein
TE	Thioesterase
C	Condensation
A	Adenylation
MEP	2C-methyl-D-erythrotol-4-phosphate
MVA	Mevalonate
SAR	Structure activity relationship

LITERATURE CITED

1. Li JWH, Vederas JC. Drug discovery and natural products: end of an era or an endless frontier? *Science*. 2009; 325:161–5. [PubMed: 19589993]
2. Harvey AL. Natural products in drug discovery. *Drug Discov Today*. 2008; 13:894–901. [PubMed: 18691670]
3. Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. *J Nat Prod*. 2007; 70:461–77. [PubMed: 17309302]
4. Demain AL, Adrio JL. Contributions of microorganisms to industrial biology. *Mol Biotechnol*. 2008; 38:41–55. [PubMed: 18060538]
5. Stephanopoulos, G.; Nielsen, J.; Aristodou, A. *Metabolic Engineering*. San Diego: Academic Press; 1998.
6. Oberhardt MA, Palsson BO, Papin JA. Applications of genome-scale metabolic reconstructions. *Mol Syst Biol*. 2009; 5:320. [PubMed: 19888215]
7. Savile CK, Janey JM, Mundorff EC, Moore JC, Tam S, et al. Biocatalytic asymmetric synthesis of chiral amines from ketones applied to sitagliptin manufacture. *Science*. 2010; 329:305–9. [PubMed: 20558668]
8. Challis GL, Hopwood DA. Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. *Proc Natl Acad Sci USA*. 2003; 100(Suppl 2):14555–61. [PubMed: 12970466]
9. Parekh, S. Strain improvement. In: Schaechter, M., editor. *The desk encyclopedia of microbiology*. San Diego: Elsevier Academic Press; 2004. p. 960-73.
10. Scherlach K, Hertweck C. Triggering cryptic natural product biosynthesis in microorganisms. *Org Biomol Chem*. 2009; 7:1753–60. [PubMed: 19590766]
11. Rokem JS, Lantz AE, Nielsen J. Systems biology of antibiotic production by microorganisms. *Nat Prod Rep*. 2007; 24:1262–87. [PubMed: 18033579]
12. Berdy J. Bioactive microbial metabolites. *J Antibiot*. 2005; 58:1–26. [PubMed: 15813176]
13. Hopwood, DA.; Kieser, T.; Bibb, M.; Buttner, M.; Chater, K. *Practical Streptomyces Genetics*. Norwich: John Innes Foundation; 2000.
14. McDaniel R, Ebert-Khosla S, Hopwood DA, Khosla C. Engineered biosynthesis of novel polyketides. *Science*. 1993; 262:1546–50. [PubMed: 8248802]
15. Ziermann R, Betlach MC. Recombinant polyketide synthesis in *Streptomyces*: engineering of improved host strains. *Biotechniques*. 1999; 26:106–10. [PubMed: 9894599]

16. Penn J, Li X, Whiting A, Latif M, Gibson T, et al. Heterologous production of daptomycin in *Streptomyces lividans*. *J Ind Microbiol Biotechnol*. 2006; 33:121–8. [PubMed: 16261359]
17. Li C, Hazzard C, Florova G, Reynolds KA. High titer production of tetracenomycins by heterologous expression of the pathway in a *Streptomyces cinnamomensis* industrial monensin producer strain. *Metab Eng*. 2009; 11:319–27. [PubMed: 19595787]
18. Pfeifer BA, Admiraal SJ, Gramajo H, Cane DE, Khosla C. Biosynthesis of complex polyketides in a metabolically engineered strain of *E. coli*. *Science*. 2001; 291:1790–2. [PubMed: 11230695]
19. Watanabe K, Rude MA, Walsh CT, Khosla C. Engineered biosynthesis of an ansamycin polyketide precursor in *Escherichia coli*. *Proc Natl Acad Sci USA*. 2003; 100:9774–8. [PubMed: 12888623]
20. Peiru S, Menzella HG, Rodriguez E, Carney J, Gramajo H. Production of the potent antibacterial polyketide erythromycin C in *Escherichia coli*. *Appl Environ Microbiol*. 2005; 71:2539–47. [PubMed: 15870344]
21. Gruenewald S, Mootz HD, Stehmeier P, Stachelhaus T. In vivo production of artificial nonribosomal peptide products in the heterologous host *Escherichia coli*. *Appl Environ Microbiol*. 2004; 70:3282–91. [PubMed: 15184122]
22. Watanabe K, Hotta K, Praseuth AP, Koketsu K, Migita A, et al. Total biosynthesis of antitumor nonribosomal peptides in *Escherichia coli*. *Nat Chem Biol*. 2006; 2:423–8. [PubMed: 16799553]
23. Mutka SC, Carney JR, Liu Y, Kennedy J. Heterologous production of epothilone C and D in *Escherichia coli*. *Biochemistry*. 2006; 45:1321–30. [PubMed: 16430229]
24. Pfeifer BA, Wang CCC, Walsh CT, Khosla C. Biosynthesis of yersiniabactin, a complex polyketide-nonribosomal peptide, using *Escherichia coli* as a heterologous host. *Appl Environ Microbiol*. 2003; 69:6698–702. [PubMed: 14602630]
25. Baltz RH. *Streptomyces* and *Saccharopolyspora* hosts for heterologous expression of secondary metabolite gene clusters. *J Ind Microbiol Biotechnol*. 2010; 37:759–72. [PubMed: 20467781]
26. Boghigian BA, Pfeifer BA. Current status, strategies, and potential for the metabolic engineering of heterologous polyketides in *Escherichia coli*. *Biotechnol Lett*. 2008; 30:1323–30. [PubMed: 18488149]
27. Challis GL. Engineering *Escherichia coli* to produce nonribosomal peptide antibiotics. *Nat Chem Biol*. 2006; 2:398–400. [PubMed: 16850013]
28. Zhang H, Wang Y, Pfeifer BA. Bacterial hosts for natural product production. *Mol Pharmaceutics*. 2008; 5:212–25.
29. Hoffmeister D, Keller NP. Natural products of filamentous fungi: enzymes, genes, and their regulation. *Nat Prod Rep*. 2007; 24:393–416. [PubMed: 17390002]
30. Manzoni M, Rollini M. Biosynthesis and biotechnological production of statins by filamentous fungi and application of these cholesterol-lowering drugs. *Appl Microbiol Biotechnol*. 2002; 58:555–64. [PubMed: 11956737]
31. Lubertozzi D, Keasling JD. Developing *Aspergillus* as a host for heterologous expression. *Biotechnol Adv*. 2009; 27:53–75. [PubMed: 18840517]
32. Bedford DJ, Schweizer E, Hopwood DA, Khosla C. Expression of a functional fungal polyketide synthase in the bacterium *Streptomyces coelicolor* A3(2). *J Bacteriol*. 1995; 177:4544–8. [PubMed: 7635840]
33. Kealey JT, Liu L, Santi DV, Betlach MC, Barr PJ. Production of a polyketide natural product in nonpolyketide-producing prokaryotic and eukaryotic hosts. *Proc Natl Acad Sci USA*. 1998; 95:505–9. [PubMed: 9435221]
34. Fujii I, Ono Y, Tada H, Gomi K, Ebizuka Y, et al. 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*. 1996; 253:1–10. [PubMed: 9003280]
35. Xu Y, Orozco R, Wijeratne EMK, Gunatilaka AAL, Stock SP, et al. Biosynthesis of the cyclooligomer depsipeptide beauvericin, a virulence factor of the entomopathogenic fungus *Beauveria bassiana*. *Chem Biol*. 2008; 15:898–907. [PubMed: 18804027]
36. Heneghan MN, Yakasai AA, Halo LM, Song Z, Bailey AM, et al. First heterologous reconstruction of a complete functional fungal biosynthetic multigene cluster. *ChemBioChem*. 2010; 11:1508–12. [PubMed: 20575135]

37. Ma SM, Li JWH, Choi JW, Zhou H, Lee KKM, et al. Complete reconstitution of a highly reducing iterative polyketide synthase. *Science*. 2009; 326:589–92. [PubMed: 19900898]
38. Schümann J, Hertweck C. Advances in cloning, functional analysis and heterologous expression of fungal polyketide synthase genes. *J Biotechnol*. 2006; 124:690–703. [PubMed: 16716432]
39. Schneider P, Misiek M, Hoffmeister D. In vivo and in vitro production options for fungal secondary metabolites. *Mol Pharmaceutics*. 2008; 5:234–42.
40. Huang Q, Roessner CA, Croteau R, Scott AI. Engineering *Escherichia coli* for the synthesis of taxadiene, a key intermediate in the biosynthesis of taxol. *Bioorg Med Chem*. 2001; 9:2237–42. [PubMed: 11553461]
41. Engels B, Dahm P, Jennewein S. Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. *Metab Eng*. 2008; 10:201–6. [PubMed: 18485776]
42. Martin VJJ, Pitera DJ, Withers ST, Newman JD, Keasling JD. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat Biotechnol*. 2003; 21:796–802. [PubMed: 12778056]
43. Ro D-K, Paradise EM, Ouellet M, Fisher KJ, Newman KL, et al. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature*. 2006; 440:940–3. [PubMed: 16612385]
44. Minami H, Kim J-S, Ikezawa N, Takemura T, Katayama T, et al. Microbial production of plant benzyloquinoline alkaloids. *Proc Natl Acad Sci USA*. 2008; 105:7393–8. [PubMed: 18492807]
45. Hawkins KM, Smolke CD. Production of benzyloquinoline alkaloids in *Saccharomyces cerevisiae*. *Nat Chem Biol*. 2008; 4:564–73. [PubMed: 18690217]
46. Horinouchi S. Combinatorial biosynthesis of plant medicinal polyketides by microorganisms. *Curr Opin Chem Biol*. 2009; 13:197–204. [PubMed: 19264534]
47. Miyahisa I, Kaneko M, Funa N, Kawasaki H, Kojima H, et al. Efficient production of (2*S*)-flavanones by *Escherichia coli* containing an artificial biosynthetic gene cluster. *Appl Gen Mol Biotechnol*. 2007; 68:498–504.
48. Gillam EMJ. Engineering cytochrome P450 enzymes. *Chem Res Toxicol*. 2008; 21:220–31. [PubMed: 18067267]
49. Leonard E, Koffas MAG. Engineering of artificial plant cytochrome P450 enzymes for synthesis of isoflavones by *Escherichia coli*. *Appl Environ Microbiol*. 2007; 73:7246–51. [PubMed: 17905887]
50. Chang MCY, Eachus RA, Trieu W, Ro D-K, Keasling JD. Engineering *Escherichia coli* for production of functionalized terpenoids using plant P450s. *Nat Chem Biol*. 2007; 3:274–7. [PubMed: 17438551]
51. Ryu Y-G, Butler MJ, Chater KF, Lee KJ. Engineering of primary carbohydrate metabolism for increased production of actinorhodin in *Streptomyces coelicolor*. *Appl Environ Microbiol*. 2006; 72:7132–9. [PubMed: 16950896]
52. Zha W, Rubin-Pitel SB, Shao Z, Zhao H. Improving cellular malonyl-CoA level in *Escherichia coli* via metabolic engineering. *Metab Eng*. 2009; 11:192–8. [PubMed: 19558964]
53. Fowler ZL, Gikandi WW, Koffas MAG. Increased malonyl coenzyme A biosynthesis by tuning the *Escherichia coli* metabolic network and its application to flavanone production. *Appl Environ Microbiol*. 2009; 75:5831–9. [PubMed: 19633125]
54. Cropp A, Chen S, Liu H, Zhang W, Reynolds KA. Genetic approaches for controlling ratios of related polyketide products in fermentation processes. *J Ind Microbiol Biotechnol*. 2001; 27:368–77. [PubMed: 11774002]
55. Reeves AR, Brikun IA, Cernota WH, Leach BI, Gonzalez MC, et al. Engineering of the methylmalonyl-CoA metabolite node of *Saccharopolyspora erythraea* for increased erythromycin production. *Metab Eng*. 2007; 9:293–303. [PubMed: 17482861]
56. Han SJ, Park SW, Kim BW, Sim SJ. Selective production of epothilone B by heterologous expression of propionyl-CoA synthetase in *Sorangium cellulosum*. *J Microbiol Biotechnol*. 2008; 18:135–7. [PubMed: 18239430]
57. Kuzuyama T. Mevalonate and nonmevalonate pathways for the biosynthesis of isoprene units. *Biosci Biotechnol Biochem*. 2002; 66:1619–27. [PubMed: 12353619]

58. Farmer WR, Liao JC. Precursor balancing for metabolic engineering of lycopene production in *Escherichia coli*. *Biotechnol Prog*. 2001; 17:57–61. [PubMed: 11170480]
59. Kajiwara S, Fraser PD, Kondo K, Misawa N. Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in *Escherichia coli*. *Biochem J*. 1997; 324:421–6. [PubMed: 9182699]
60. Kim SW, Keasling JD. Metabolic engineering of the nonmevalonate isopentenyl diphosphate synthesis pathway in *Escherichia coli* enhances lycopene production. *Biotechnol Bioeng*. 2001; 72:408–15. [PubMed: 11180061]
61. Pitera DJ, Paddon CJ, Newman JD, Keasling JD. Balancing a heterologous mevalonate pathway for improved isoprenoid production in *Escherichia coli*. *Metab Eng*. 2007; 9:193–207. [PubMed: 17239639]
62. Shiba Y, Paradise EM, Kirby J, Ro D-K, Keasling JD. Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids. *Metab Eng*. 2007; 9:160–8. [PubMed: 17196416]
63. Knaggs AR. The biosynthesis of shikimate metabolites. *Nat Prod Rep*. 1999; 16:525–60. [PubMed: 10467741]
64. Thykaer J, Nielsen J, Wohlleben W, Weber T, Gutknecht M, et al. Increased glycopeptide production after overexpression of shikimate pathway genes being part of the balhimycin biosynthetic gene cluster. *Metab Eng*. 2010; 12:455–61. [PubMed: 20570618]
65. Yanai K, Murakami T, Bibb M. Amplification of the entire kanamycin biosynthetic gene cluster during empirical strain improvement of *Streptomyces kanamyceticus*. *Proc Natl Acad Sci USA*. 2006; 103:9661–6. [PubMed: 16766657]
66. Smith DJ, Bull JH, Edwards J, Turner G. Amplification of the isopenicillin N synthetase gene in a strain of *Penicillium chrysogenum* producing high levels of penicillin. *Mol Gen Genet*. 1989; 216:492–7. [PubMed: 2501652]
67. Peschke U, Schmidt H, Zhang HZ, Piepersberg W. Molecular characterization of the lincomycin-production gene cluster of *Streptomyces lincolnensis* 78–11. *Mol Microbiol*. 1995; 16:1137–56. [PubMed: 8577249]
68. Anthony JR, Anthony LC, Nowroozi F, Kwon G, Newman JD, et al. 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*. 2009; 11:13–9. [PubMed: 18775787]
69. Keasling JD. Gene-expression tools for the metabolic engineering of bacteria. *Trends Biotechnol*. 1999; 17:452–60. [PubMed: 10511704]
70. Leonard E, Ajikumar PK, Thayer K, Xiao W-H, Mo JD, et al. Combining metabolic and protein engineering of a terpenoid biosynthetic pathway for overproduction and selectivity control. *Proc Natl Acad Sci USA*. 2010; 107:13654–9. [PubMed: 20643967]
71. Kennedy J, Turner G. δ -(L- α -Aminoadipyl)-L-cysteiny-D-valine synthetase is a rate limiting enzyme for penicillin production in *Aspergillus nidulans*. *Mol Gen Genet*. 1996; 253:189–97. [PubMed: 9003303]
72. Fernández-Cañón JM, Peñalva MA. Overexpression of two penicillin structural genes in *Aspergillus nidulans*. *Mol Gen Genet*. 1995; 246:110–8. [PubMed: 7823906]
73. Veenstra AE, van Solingen P, Bovenberg RAL, van der Voort LHM. Strain improvement of *Penicillium chrysogenum* by recombinant DNA techniques. *J Biotechnol*. 1991; 17:81–90. [PubMed: 1367496]
74. Malla S, Niraula NP, Liou K, Sohng JK. Enhancement of doxorubicin production by expression of structural sugar biosynthesis and glycosyltransferase genes in *Streptomyces peucetius*. *J Biosci Bioeng*. 2009; 108:92–8. [PubMed: 19619853]
75. Malla S, Niraula NP, Liou K, Sohng JK. Self-resistance mechanism in *Streptomyces peucetius*: Overexpression of *draA*, *draB* and *draC* for doxorubicin enhancement. *Microbiol Res*. 2010; 165:259–67. [PubMed: 19651502]
76. Chen Y, Smanski M, Shen B. Improvement of secondary metabolite production in *Streptomyces* by manipulating pathway regulation. *Appl Microbiol and Biotechnol*. 2010; 86:19–25. [PubMed: 20091304]

77. Chen Y, Wendt-Pienkowski E, Shen B. 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.* 2008; 190:5587–96. [PubMed: 18556785]
78. Lombo F, Brana AF, Mendez C, Salas JA. 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.* 1999; 181:642–7. [PubMed: 9882681]
79. Sakai K, Kinoshita H, Shimizu T, Nihira T. Construction of a citrinin gene cluster expression system in heterologous *Aspergillus oryzae*. *J Biosci Bioeng.* 2008; 106:466–72. [PubMed: 19111642]
80. Bergmann S, Schumann J, Scherlach K, Lange C, Brakhage AA, et al. Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*. *Nat Chem Biol.* 2007; 3:213–7. [PubMed: 17369821]
81. Chiang YM, Szewczyk E, Davidson AD, Keller N, Oakley BR, et al. A gene cluster containing two fungal polyketide synthases encodes the biosynthetic pathway for a polyketide, asperfuranone, in *Aspergillus nidulans*. *J Am Chem Soc.* 2009; 131:2965–70. [PubMed: 19199437]
82. Smanski MJ, Peterson RM, Rajski SR, Shen B. Engineered *Streptomyces platensis* strains that overproduce antibiotics platensimycin and platencin. *Antimicrob Agents Chemother.* 2009; 53:1299–304. [PubMed: 19164156]
83. Alper H, Fischer C, Nevoigt E, Stephanopoulos G. Tuning genetic control through promoter engineering. *Proc Natl Acad Sci USA.* 2005; 102:12678–83. [PubMed: 16123130]
84. Nevoigt E, Kohnke J, Fischer CR, Alper H, Stahl U, et al. Engineering of promoter replacement cassettes for fine-tuning of gene expression in *Saccharomyces cerevisiae*. *Appl Environ Microbiol.* 2006; 72:5266–73. [PubMed: 16885275]
85. Pflieger BF, Pitera DJ, Smolke CD, Keasling JD. Combinatorial engineering of intergenic regions in operons tunes expression of multiple genes. *Nat Biotechnol.* 2006; 24:1027–32. [PubMed: 16845378]
86. Salis HM, Mirsky EA, Voigt CA. Automated design of synthetic ribosome binding sites to control protein expression. *Nat Biotechnol.* 2009; 27:946–50. [PubMed: 19801975]
87. Kizer L, Pitera DJ, Pflieger BF, Keasling JD. Application of functional genomics to pathway optimization for increased isoprenoid production. *Appl Environ Microbiol.* 2008; 74:3229–41. [PubMed: 18344344]
88. Fox RJ, Huisman GW. Enzyme optimization: moving from blind evolution to statistical exploration of sequence function space. *Trends Biotechnol.* 2008; 26:132–8. [PubMed: 18222559]
89. Wang, Cw; Oh, MK.; Liao, JC. Directed evolution of metabolically engineered *Escherichia coli* for carotenoid production. *Biotechnol Prog.* 2000; 16:922–6. [PubMed: 11101317]
90. Paradise EM, Kirby J, Chan R, Keasling JD. Redirection of flux through the FPP branch-point in *Saccharomyces cerevisiae* by down-regulating squalene synthase. *Biotechnol Bioeng.* 2008; 100:371–8. [PubMed: 18175359]
91. Xie X, Wong WW, Tang Y. Improving simvastatin bioconversion in *Escherichia coli* by deletion of *bioH*. *Metab Eng.* 2007; 9:379–86. [PubMed: 17625941]
92. Lomovskaya N, Otten SL, Doi-Katayama Y, Fonstein L, Liu X-C, et al. Doxorubicin overproduction in *Streptomyces peuceitii*: cloning and characterization of the *dnrU* ketoreductase and *dnrV* genes and the *doxA* cytochrome P-450 hydroxylase gene. *J Bacteriol.* 1999; 181:305–18. [PubMed: 9864344]
93. Chemler JA, Fowler ZL, McHugh KP, Koffas MAG. Improving NADPH availability for natural product biosynthesis in *Escherichia coli* by metabolic engineering. *Metab Eng.* 2010; 12:96–104. [PubMed: 19628048]
94. Sung, BH.; Lee, JH.; Kim, SC. *Escherichia coli* genome engineering and minimization for the construction of a bioengine. In: Lee, SY., editor. *Systems Biology and Biotechnology of Escherichia coli*. London: Springer Science_Business Media; 2009.
95. Komatsu M, Uchiyama T, Omura S, Cane DE, Ikeda H. Genome-minimized *Streptomyces* host for the heterologous expression of secondary metabolism. *Proc Natl Acad Sci USA.* 2010; 107:2646–51. [PubMed: 20133795]

96. Floss HG. Combinatorial biosynthesis - Potential and problems. *J Biotechnol.* 2006; 124:242–57. [PubMed: 16414140]
97. Baltz RH. Molecular engineering approaches to peptide, polyketide and other antibiotics. *Nat Biotechnol.* 2006; 24:1533–40. [PubMed: 17160059]
98. Madduri K, Kennedy J, Rivola G, Inventi-Solari A, Filippini S, et al. Production of the antitumor drug epirubicin (4'-epidoxorubicin) and its precursor by a genetically engineered strain of *Streptomyces peucetius*. *Nat Biotechnol.* 1998; 16:69–74. [PubMed: 9447597]
99. Fischbach MA, Walsh CT, Clardy J. The evolution of gene collectives: How natural selection drives chemical innovation. *Proc Natl Acad Sci USA.* 2008; 105:4601–8. [PubMed: 18216259]
100. Mendes M, Recio E, Fouces R, Luiten R, Martin J, et al. Engineered biosynthesis of novel polyenes: a pimaricin derivative produced by targeted gene disruption in *Streptomyces natalensis*. *Chem Biol.* 2001; 8:635–44. [PubMed: 11451665]
101. Remsing LL, Gonzalez AM, Nur-e-Alam M, Fernandez-Lozano MJ, Brana AF, et al. 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.* 2003; 125:5745–53. [PubMed: 12733914]
102. Ito T, Roongsawang N, Shirasaka N, Lu W, Platt PM, et al. Deciphering pactamycin biosynthesis and engineered production of new pactamycin analogues. *ChemBioChem.* 2009; 10:2253–65. [PubMed: 19670201]
103. Ju J, Li W, Yuan Q, Peters NR, Hoffmann FM, et al. Functional characterization of *ttmM* unveils new tautomycin analogs and insight into tautomycin biosynthesis and activity. *Org Lett.* 2009; 11:1639–42. [PubMed: 19281218]
104. Zhang M-Q, Gaisser S, Nur-E-Alam M, Sheehan LS, Vousden WA, et al. Optimizing natural products by biosynthetic engineering: discovery of nonquinone Hsp90 Inhibitors. *J Med Chem.* 2008; 51:5494. [PubMed: 18800759]
105. Tang L, Chung L, Carney JR, Starks CM, Licari P, et al. Generation of new epothilones by genetic engineering of a polyketide synthase in *Myxococcus xanthus*. *J Antibiot.* 2005; 58:178–84. [PubMed: 15895525]
106. Power P, Dunne T, Murphy B, Nic Lochlainn L, Rai D, et al. Engineered synthesis of 7-oxo- and 15-deoxy-15-oxo-amphotericins: insights into structure-activity relationships in polyene antibiotics. *Chem Biol.* 2008; 15:78–86. [PubMed: 18215775]
107. Khaw L, Bohm G, Metcalfe S, Staunton J, Leadlay P. Mutational biosynthesis of novel rapamycins by a strain of *Streptomyces hygroscopicus* NRRL 5491 disrupted in *rapL*, encoding a putative lysine cyclodeaminase. *J Bacteriol.* 1998; 180:809. [PubMed: 9473033]
108. Weist S, Bister B, Puk O, Bischoff D, Pelzer S, et al. Fluorobalhimycin-A new chapter in glycopeptide antibiotic research. *Angew Chem Int Edit.* 2002; 41:3383–5.
109. Galm U, Dessoy MA, Schmidt J, Wessjohann LA, Heide L. In vitro and in vivo production of new aminocoumarins by a combined biochemical, genetic, and synthetic approach. *Chem Biol.* 2004; 11:173–83. [PubMed: 15123279]
110. Li SM, Heide L. New aminocoumarin antibiotics from genetically engineered *Streptomyces* strains. *Curr Med Chem.* 2005; 12:419–27. [PubMed: 15720250]
111. Kim W, Lee JS, Lee D, Cai XF, Shin JC, et al. Mutasynthesis of geldanamycin by the disruption of a gene producing starter unit: generation of structural diversity at the benzoquinone ring. *ChemBioChem.* 2007; 8:1491–4. [PubMed: 17661303]
112. Menzella HG, Tran TT, Carney JR, Lau-Wee J, Galazzo J, et al. Potent non-benzoquinone ansamycin heat shock protein 90 inhibitors from genetic engineering of *Streptomyces hygroscopicus*. *J Med Chem.* 2009; 52:1518–21. [PubMed: 19231864]
113. Eustaquio AS, Moore BS. Mutasynthesis of fluorosalinosporamide, a potent and reversible inhibitor of the proteasome. *Angew Chem Int Ed.* 2008; 47:3936–8.
114. Eustaquio A, O'Hagan D, Moore B. Engineering fluorometabolite production: fluorinase expression in *Salinispora tropica* yields fluorosalinosporamide. *J Nat Prod.* 2010; 73:378–82. [PubMed: 20085308]

115. Runguphan W, Maresh JJ, O'Connor SE. Silencing of tryptamine biosynthesis for production of nonnatural alkaloids in plant culture. *Proc Natl Acad Sci USA*. 2009; 106:13673–8. [PubMed: 19666570]
116. Weissman KJ. Mutasynthesis - uniting chemistry and genetics for drug discovery. *Trends Biotechnol*. 2007; 25:139–42. [PubMed: 17306390]
117. Hopwood DA, Malpartida F, Kieser HM, Ikeda H, Duncan J, et al. Production of 'hybrid' antibiotics by genetic engineering. *Nature*. 1985; 314:642–4. [PubMed: 3857464]
118. de Boer AL, Schmidt-Dannert C. Recent efforts in engineering microbial cells to produce new chemical compounds. *Curr Opin Chem Biol*. 2003; 7:273–8. [PubMed: 12714061]
119. Pfeifer BA, Khosla C. Biosynthesis of polyketides in heterologous hosts. *Microbiol Mol Biol Rev*. 2001; 65:106–18. [PubMed: 11238987]
120. Cane DE, Walsh CT, Khosla C. Harnessing the biosynthetic code: combinations, permutations, and mutations. *Science*. 1998; 282:63–8. [PubMed: 9756477]
121. Moyne AL, Cleveland TE, Tuzun S. Molecular characterization and analysis of the operon encoding the antifungal lipopeptide bacillomycin D. *FEMS Microbiol Lett*. 2004; 234:43–9. [PubMed: 15109718]
122. Reeves CD, Ward SL, Revill WP, Suzuki H, Marcus M, et al. Production of hybrid 16-membered macrolides by expressing combinations of polyketide synthase genes in engineered *Streptomyces fradiae* hosts. *Chem Biol*. 2004; 11:1465–72. [PubMed: 15489173]
123. McDaniel R, Thamchaipenet A, Gustafsson C, Fu H, Betlach M, et al. Multiple genetic modifications of the erythromycin polyketide synthase to produce a library of novel "unnatural" natural products. *Proc Natl Acad Sci USA*. 1999; 96:1846–51. [PubMed: 10051557]
124. Rascher A, Hu ZH, Viswanathan N, Schirmer A, Reid R, et al. Cloning and characterization of a gene cluster for geldanamycin production in *Streptomyces hygroscopicus* NRRL 3602. *FEMS Microbiol Lett*. 2003; 218:223–30. [PubMed: 12586396]
125. Patel K, Piagentini M, Rascher A, Tian ZQ, Buchanan GO, et al. Engineered biosynthesis of geldanamycin analogs for Hsp90 inhibition. *Chem Biol*. 2004; 11:1625–33. [PubMed: 15610846]
126. Gaisser S, Kellenberger L, Kaja AL, Weston AJ, Lill RE, et al. Direct production of ivermectin-like drugs after domain exchange in the avermectin polyketide synthase of *Streptomyces avermitilis* ATCC31272. *Org Biomol Chem*. 2003; 1:2840–7. [PubMed: 12968333]
127. Miao V, Coeffet-Le Gal MF, Nguyen K, Brian P, Penn J, et al. Genetic engineering in *Streptomyces roseosporus* to produce hybrid lipopeptide antibiotics. *Chem Biol*. 2006; 13:269–76. [PubMed: 16638532]
128. Nguyen KT, Ritz D, Gu JQ, Alexander D, Chu M, et al. Combinatorial biosynthesis of novel antibiotics related to daptomycin. *Proc Natl Acad Sci USA*. 2006; 103:17462–7. [PubMed: 17090667]
129. Coeffet-Le Gal MF, Thurston L, Rich P, Miao V, Baltz RH. Complementation of daptomycin *dptA* and *dptD* deletion mutations in trans and production of hybrid lipopeptide antibiotics. *Microbiology*. 2006; 152:2993–3001. [PubMed: 17005980]
130. Nguyen KT, He XW, Alexander DC, Li C, Gu JQ, et al. Genetically engineered lipopeptide antibiotics related to A54145 and daptomycin with improved properties. *Antimicrob Agents Chemother*. 2010; 54:1404–13. [PubMed: 20086142]
131. Rodriguez E, McDaniel R. Combinatorial biosynthesis of antimicrobials and other natural products. *Curr Opin Microbiol*. 2001; 4:526–34. [PubMed: 11587928]
132. Baltz RH. Daptomycin: mechanisms of action and resistance, and biosynthetic engineering. *Curr Opin Chem Biol*. 2009; 13:144–51. [PubMed: 19303806]
133. Park SR, Han AR, Ban YH, Yoo YJ, Kim EJ, et al. Genetic engineering of macrolide biosynthesis: past advances, current state, and future prospects. *Appl Environ Microbiol*. 2010; 85:1227–39.
134. Strieker M, Marahiel MA. The structural diversity of acidic lipopeptide antibiotics. *ChemBioChem*. 2009; 10:607–16. [PubMed: 19156787]
135. Weissman KJ, Leadlay PF. Combinatorial biosynthesis of reduced polyketides. *Nat Rev Microbiol*. 2005; 3:925–36. [PubMed: 16322741]

136. Mendez C, Salas JA. Altering the glycosylation pattern of bioactive compounds. *Trends Biotechnol.* 2001; 19:449–56. [PubMed: 11602309]
137. Luzhetskyy A, Bechthold A. It works: combinatorial biosynthesis for generating novel glycosylated compounds. *Mol Microbiol.* 2005; 58:3–5. [PubMed: 16164544]
138. Olano C, Mendez C, Salas JA. Post-PKS tailoring steps in natural product-producing actinomycetes from the perspective of combinatorial biosynthesis. *Nat Prod Rep.* 2010; 27:571–616. [PubMed: 20352666]
139. Flatman RH, Eustaquio A, Li SM, Heide L, Maxwell A. Structure-activity relationships of aminocoumarin-type gyrase and topoisomerase IV inhibitors obtained by combinatorial biosynthesis. *Antimicrob Agents Chemother.* 2006; 50:1136–42. [PubMed: 16569821]
140. Sanchez C, Zhu LL, Brana AF, Salas AP, Rohr J, et al. Combinatorial biosynthesis of antitumor indolocarbazole compounds. *Proc Natl Acad Sci USA.* 2005; 102:461–6. [PubMed: 15625109]
141. Sanchez C, Salas AP, Brana AF, Palomino M, Pineda-Lucena A, et al. Generation of potent and selective kinase inhibitors by combinatorial biosynthesis of glycosylated indolocarbazoles. *Chem Commun.* 2009:4118–20.
142. Werneburg M, Busch B, He J, Richter ME, Xiang L, et al. Exploiting enzymatic promiscuity to engineer a focused library of highly selective antifungal and antiproliferative aureothin analogues. *J Am Chem Soc.* 2010; 132:10407–13. [PubMed: 20662518]
143. Horinouchi S. Combinatorial biosynthesis of non-bacterial and unnatural flavonoids, stilbenoids and curcuminoids by microorganisms. *J Antibiot.* 2008; 61:709–28. [PubMed: 19194030]
144. Katsuyama Y, Funa N, Miyahisa I, Horinouchi S. Synthesis of unnatural flavonoids and stilbenes by exploiting the plant biosynthetic pathway in *Escherichia coli*. *Chem Biol.* 2007; 14:613–21. [PubMed: 17584609]
145. Leonard E, Runguphan W, O'Connor S, Prather KJ. Opportunities in metabolic engineering to facilitate scalable alkaloid production. *Nat Chem Biol.* 2009; 5:292–300. [PubMed: 19377455]
146. Williams GJ, Goff RD, Zhang C, Thorson JS. Optimizing glycosyltransferase specificity via “hot spot” saturation mutagenesis presents a catalyst for novobiocin glycorandomization. *Chem Biol.* 2008; 15:393–401. [PubMed: 18420146]
147. Williams GJ, Zhang C, Thorson JS. Expanding the promiscuity of a natural-product glycosyltransferase by directed evolution. *Nat Chem Biol.* 2007; 3:657–62. [PubMed: 17828251]
148. Chen S, Galan MC, Coltharp C, O'Connor SE. Redesign of a central enzyme in alkaloid biosynthesis. *Chem Biol.* 2006; 13:1137–41. [PubMed: 17113995]
149. Runguphan W, O'Connor SE. Metabolic reprogramming of periwinkle plant culture. *Nat Chem Biol.* 2009; 5:151–3. [PubMed: 19151732]
150. Schmidt-Dannert C, Umeno D, Arnold FH. Molecular breeding of carotenoid biosynthetic pathways. *Nat Biotechnol.* 2000; 18:750–3. [PubMed: 10888843]
151. Yoshikuni Y, Ferrin TE, Keasling JD. Designed divergent evolution of enzyme function. *Nature.* 2006; 440:1078–82. [PubMed: 16495946]
152. Hans M, Hornung A, Dziarnowski A, Cane DE, Khosla C. Mechanistic analysis of acyl transferase domain exchange in polyketide synthase modules. *J Am Chem Soc.* 2003; 125:5366–74. [PubMed: 12720450]
153. Hunziker D, Yu TW, Hutchinson CR, Floss HG, Khosla C. Primer unit specificity in rifamycin biosynthesis principally resides in the later stages of the biosynthetic pathways. *J Am Chem Soc.* 1998; 120:1092–3.
154. Zhou Z, Lai JR, Walsh CT. Directed evolution of aryl carrier proteins in the enterobactin synthetase. *Proc Natl Acad Sci USA.* 2007; 104:11621–6. [PubMed: 17606920]
155. Fischbach MA, Lai JR, Roche ED, Walsh CT, Liu DR. Directed evolution can rapidly improve the activity of chimeric assembly-line enzymes. *Proc Natl Acad Sci USA.* 2007; 104:11951–6. [PubMed: 17620609]

Figure 1.
Major classes of natural products. PK – polyketide, NRP – non-ribosomal peptide.

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 *rapAT2* and *rapDH/KR4* domains from rapamycin synthase (RAPS) (123). **B.** exchange of daptomycin Dpt module 11 and subunit DptD with corresponding module and subunit from A54145 pathway (LptABCD) (128). Loading module and subunit DptA are not shown as it is unchanged and the parent daptomycin structure is shown in Figure 1. **C.** swapping of tailoring genes *clo-hal* (halogenase) and *novO* (methyltransferase) between clorobiocin and novobiocin pathways (110).