Metabolic engineering of microorganisms: general strategies and drug production

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Many drugs and drug precursors found in natural organisms are rather difficult to synthesize chemically and to extract in large amounts. Metabolic engineering is playing an increasingly important role in the production of these drugs and drug precursors. This is typically achieved by establishing new metabolic pathways leading to the product formation, and enforcing or removing the existing metabolic pathways toward enhanced product formation. Recent advances in system biology and synthetic biology are allowing us to perform metabolic engineering at the whole cell level, thus enabling optimal design of a microorganism for the efficient production of drugs and drug precursors. In this review, we describe the general strategies for the metabolic engineering of microorganisms for the production of drugs and drug precursors. As successful examples of metabolic engineering, the approaches taken toward strain development for the production of artemisinin, an antimalarial drug, and benzylisoquinoline alkaloids, a family of antibacterial and anticancer drugs, are described in detail. Also, systems metabolic engineering of *Escherichia coli* for the production of L-valine, an important drug precursor, is showcased as an important strategy of future metabolic engineering effort.

Introduction

Since the advent of recombinant DNA technology, genetic engineering of cells, particularly microorganisms, has been successfully practiced for the development of strains capable of overproducing recombinant proteins and small molecule chemicals. For the latter, strategies beyond simple genetic engineering are often required as they are synthesized through multiple intracellular reactions, which are further complicated by various factors including cofactor balance and regulatory circuits. Metabolic engineering can be defined as purposeful modification of cellular metabolism using recombinant DNA and other molecular biological techniques [1]. Metabolic engineering considers metabolic and cellular system as an entirety and accordingly allows manipulation of the system with consideration of the efficiency of overall bioprocess, which distinguishes itself from simple genetic engineering [1–3]. Furthermore, metabolic engineering is advantageous in several aspects, compared to simple genetic engineering or random mutagenesis, since it allows defined engineering of the cell, thus avoiding unnecessary changes to the cell and allowing further engineering if necessary. Among the many successful examples of metabolic engineering, recent reports on the efficient production of L-valine [4], L-threonine [5], lycopene [6], antimalarial drug precursor [7], and benzylisoquinoline alkaloids [8] represent how metabolic engineering can be performed to achieve desired goals.

Like other industrially useful chemicals, drugs have also been the major target of metabolic engineering (Table 1). Plant secondary metabolites that are of medicinal value, such as artemisinic acid [7], taxol precursor [9], and benzylisoquinoline alkaloids [8] have been successfully produced by metabolically

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TABLE 1

Drugs and drug precursors produced by metabolic engineering of microorganisms

Application	Drugs/drug precursors	Production host	Engineering approach	Refs
Antibiotics	A novel amidated polyketide	*Streptomyces coelicolor*	Heterologous coexpression of amidotransferase OxyD with minimal oxytetracycline polyketide synthase in *S. coelicolor*.	[55]
Antibiotics	Clavulanic acid	*Streptomyces clavuligerus*	Knockout of *gap1* and *gap2* and addition of arginine in the medium to improve the drug precursors	[56]
Antibiotics	Daptomycin	*Streptomyces lividans*	Heterologous production of daptomycin in *S. lividans*, inactivation of actinorhodin, and optimization of the medium by adding additional phosphate	[51]
Antibiotics	Daptomycin derivatives	*Streptomyces roseosporus*	Use of λ-Red-medicated recombination to exchange single or several modules in the subunit of the non-ribosomal peptide synthase	[16]
Antibiotics	Erythromycin A	*Saccharopolyspora erythraea*	Overexpression of *eryK* and *eryG* with copy number ratio of 3:2	[53]
Antibiotics	Fosfomycin	*Streptomyces lividans*	Cloning of a complete fosfomycin biosynthetic cluster from *Streptomyces fradiae* and its heterologous production in *S. lividans*	[57]
Antibiotics	Lantibiotic subtilin	*Bacillus subtilis*	Overexpression of subtilin self-protection genes *spaIFEG* and deletion of a repressor of subtilin gene AbrB	[58]
Antibiotics	Macrolide 6-deoxyerythromycin D	*Escherichia coli*	Heterologous production of 6-deoxyerythromycin D in *E. coli* and several generations of activity-based screening assay for further evolution	[59]
Antibiotics	Magnoflorine and (*S*)-scoulerine	*Escherichia coli* and *Saccharomyces cerevisiae*	Heterologous production of benzylisoquinoline alkaloids in the co-culture of *E. coli* and *S. cerevisiae* using microbial and plant genes	[8]
Antibiotics	Novobiocin derivatives	*Streptomyces coelicolor*	Engineering of novobiocin biosynthetic gene cluster in *E. coli* using λ-Red-medicated recombination and its expression in *S. coelicolor* along with coexpression of the halogenase Clo-hal	[60]
Antibiotics	Tylactone	*Streptomyces venezuelae*	Heterologous expression of tylosin polyketides synthase in *S. venezuelael* and provision of precursors in the medium	[61]
Anticancer, antiviral, and antimicrobial	Valencene, cubebol, and patchoulol	*Saccharomyces cerevisiae*	Heterologous expression of plant sesquiterpenes biosynthetic genes, downregulation of ERG9 and addition of methionine to the medium	[62]
Anticancer	Torulene and novel carotenoids	*Escherichia coli*	Extension of *in vitro* evolved metabolic pathways with various downstream enzymes for novel carotenoids	[63]
Antimicrobial and anticancer	Carvone	*Escherichia coli*	Inducible expression of heterologous genes for the biosynthesis of (-)-carvone	[64]
Antioxidant	Astaxanthin	*Escherichia coli*	Overexpression of isopentenyl diphosphate isomerase from *E. coli* and geranylgeranyl diphosphate synthase from *Archaeoglobus fulgidus*, and heterologous expression of the gene cluster crtBIYZW from *Agrobacterium aurantiacum*	[65]
Antioxidant	Lycopene	*Escherichia coli*	Combination of knockout targets identified by flux balance analysis and screening of a global transposon library	[6]

TABLE 1 (*Continued*)

Application	Drugs/drug precursors	Production host	Engineering approach	Refs
Antioxidant	Lycopene	***Escherichia coli***	Heterologous expression of carotenoid genes from *Pantoea agglomerans* or *Pantoea ananatis*	[66]
Antiparasitic	8-Hydroxycadinene and artemisinic acid (precursor of gossypol and artemisinin, respectively)	*Escherichia coli*	Heterologous expression of plant-derived cytochrome P450s	[67]
Antiparasitic	Amorphadiene (precursor of antimalarial drug artemisinin)	*Escherichia coli*	Heterologous expression of mevalonate pathway from *S. cerevisiae* and codon-optimized amorphadiene synthase	[27]
Antiparasitic	Artemisinic acid (precursor of antimalarial drug artemisinin)	*Saccharomyces cerevisiae*	Overexpression of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase and *upc*2-1, a global transcriptional factor of the sterol biosynthesis, for increased farnesyl pyrophosphate precursor and heterologous expression of amorphadiene synthase and amorphadiene-oxiding enzyme with its redox partner, all isolated from *A. annua*	[7]
Antiparasitic (derivative of avermectin)	Ivermectin	*Streptomyces avermitilis*	Replacement of domains of module 2 from the avermectin polyketides synthase in *S. avermitilis* Olm73-12 with those from module 4 of the pikromycin polyketides synthase in *S. venezuelae* ATCC 15439	[68]
Antitumor	Echinomycin	*Escherichia coli*	Heterologous expression of echinomycin gene cluster in *E. coli*	[69]
Antitumor	Indolocarbazole compounds	*Streptomyces albus*	Heterologous expression of different combinations of rebeccamycin biosynthetic genes in *S. albus*	[70]
Antitumor	Taxadien-5α-acetoxy-10β-ol (precursor of Taxol)	*Saccharomyces cerevisiae*	Heterologous expression of eight of taxoid biosynthetic genes	[9]
Antiviral	Shikimic acid (starting material for the synthesis of neuraminidase inhibitor GS4104)	*Escherichia coli*	Blockage of aromatic amino acid pathway after the production of shikimic acid, amplification of carbon flux toward the shikimic acid production and additional engineering for optimizing strain performance	[71]
Antiviral	Shikimic acid (starting material for the synthesis of neuraminidase inhibitor GS4104)	*Escherichia coli*	Heterologous expression of glucose facilitator protein and glucose kinase, both from *Zymomonas mobilis*	[72]
Blood-related agents	Human α-hemoglobin	*Escherichia coli*	Coexpression of α-hemoglobin with α-hemoglobin-stabilizing molecular chaperone	[73]
Cardiovascular disease	Lovastatin (Cholesterol-lowering drug)	*Aspergillus terreus*	Statistical analysis of the transcriptional and metabolite profiles to identify key genes whose manipulation influences lovastatin production	[30]
Cardiovascular disease	Nicotianamine (antihypertensive)	*Saccharomyces cerevisiae*	Heterologous expression of the Arabidopsis AtNAS2 gene in *Saccharomyces cerevisiae*	[74]
Hormone	Gonadotropin-releasing hormone	*Escherichia coli*	Heterologous expression of the recombinant gonadotropin-releasing hormone in *E. coli* using a T7 RNA polymerase-based expression system and evaluation of various culture conditions on the plasmid stability and the product yield	[75]
Hormone	Human growth hormone	*Escherichia coli*	Activation of the promoter lambda PL by temperature shift for production of human growth hormone without contaminants	[76]

TABLE 1 (*Continued*)

Application	Drugs/drug precursors	Production host	Engineering approach	Refs
Hormone	Human parathyroid hormone	***Escherichia coli***	Using recombinant *E. coli* strain BL21 (DE3) harboring the plasmid pET32aBI1 encoding the fusion gene of thioredoxin and human parathyroid hormone	[77]
Immunological agents	Human interferon-gamma	*Escherichia coli*	Expression by using the pEMR vector that contains the Tat-dependent modified penicillin acylase signal peptide (mSPpac) by the Tat translocation pathway	[78]
Immunological agents	Human interleukin-3	*Bacillus subtilis*	Development of host-vector system and a multicopy plasmid with strong promoters and signal sequences	[41]
Immunological agents	IgG antibodies	*Escherichia coli*	Expression of IgG antibodies in the periplasm of *E. coli* and their combination with Fc-binding protein anchored in the inner membrane	[12]

engineered microorganisms. Production of human insulin by recombinant *Escherichia coli* [10] is considered as an outcome of straightforward recombinant DNA technology, but more complex proteins of great therapeutic values can also be produced by metabolic and cellular engineering of microorganisms [11,12]. Development of structurally and functionally diverse antibiotics by metabolic engineering is of great importance to fight against emerging drug-resistant pathogens [13,14]. In this paper, general strategies of metabolic engineering for drug production are described with relevant examples. Also, we suggest the approaches of employing metabolic engineering to develop new drugs and efficiently produce them.

Drugs from engineered microorganisms

Microorganisms as drug factory

Many chemicals and biological molecules that have been used as drugs are found in microorganisms, plants, and animals. As these drugs are synthesized in only minute amounts, it is difficult to obtain them in suitable amounts. This is where metabolic engineering comes into play. Recent advances in our understanding on the metabolic pathways for the synthesis of these drugs together with the development of various genetic and analytical tools have enabled more systematic and rigorous engineering of microorganisms for enhanced drug production (Figure 1). Drug production by metabolically engineered microorganisms has several advantages over total chemical synthesis or extraction from natural resources. Chemicals that are used as drugs usually have complex structures including chirality that are rather difficult to synthesize chemically. Also, extraction of medically valuable compounds from natural resources is usually inefficient and might cause negative impacts on the environment by diminishing the resources and polluting the extraction site [15]. On the contrary, drugs can be produced by microbial fermentation from relatively inexpensive substrates in a controlled and consistent manner. Much rapid growth of microbial cells compared with higher organisms is another obvious advantage. Furthermore, metabolic engineering of microorganisms can be performed more easily than mammalian and plant cells, which allows modification of metabolic pathways for the production of structurally more diverse analogs with potent biological activities, as in the cases of polyketides and non-ribosomal peptides [16]. These advantages are key driving forces for the microbial production of drugs and drug precursors.

Biosynthesis of drugs and drug precursors through metabolic engineering

Although production of drugs at their final forms may be most desirable, biosynthesis of drug precursors is also favored experimentally and economically in several cases. If there are ways of performing chemical conversion of the microbially produced drug precursor to its final form, production of the precursor is already valuable. Artemisinic acid produced in metabolically engineered *Saccharomyces cerevisiae* would be one of these cases; it is a precursor of antimalarial drug artemisinin, and can be chemically converted to the final form in two steps of reduction and oxidation. Thus, highly efficient production of artemisinin is possible using the biologically produced precursor [15]. In some cases, drug precursors themselves might also have other industrial or medicinal values, justifying their production on large scale. For example, L-valine is an essential amino acid that has important applications in food industry, including diet food supplement and health beverage, and animal feed industry, and is also a precursor of several antibiotics, monensin [17], cervimycin C [18], and valanimycin [19].

High impact of microbial metabolic engineering toward the biosynthesis of drug precursors is well illustrated by the recent development of microbial systems that allow production of various benzylisoquinoline alkaloids (Figure 2a) [8]. Benzylisoquinoline alkaloids are plant-derived secondary metabolites that have a wide range of medicinal applications, such as analgesic compounds, antibacterial agents, and antimalarial and anticancer drugs. For this, (*S*)-reticuline, a key intermediate for the biosynthesis of various benzylisoquinoline alkaloids, was first produced using *E. coli*, which was metabolically engineered with microbial and plant genes. In the newly constructed pathway in *E. coli*, monoamine oxidase (MAO) converts dopamine into 3,4-dihydroxyphenylacetaldehyde (3,4-DHPAA), and norcoclaurine

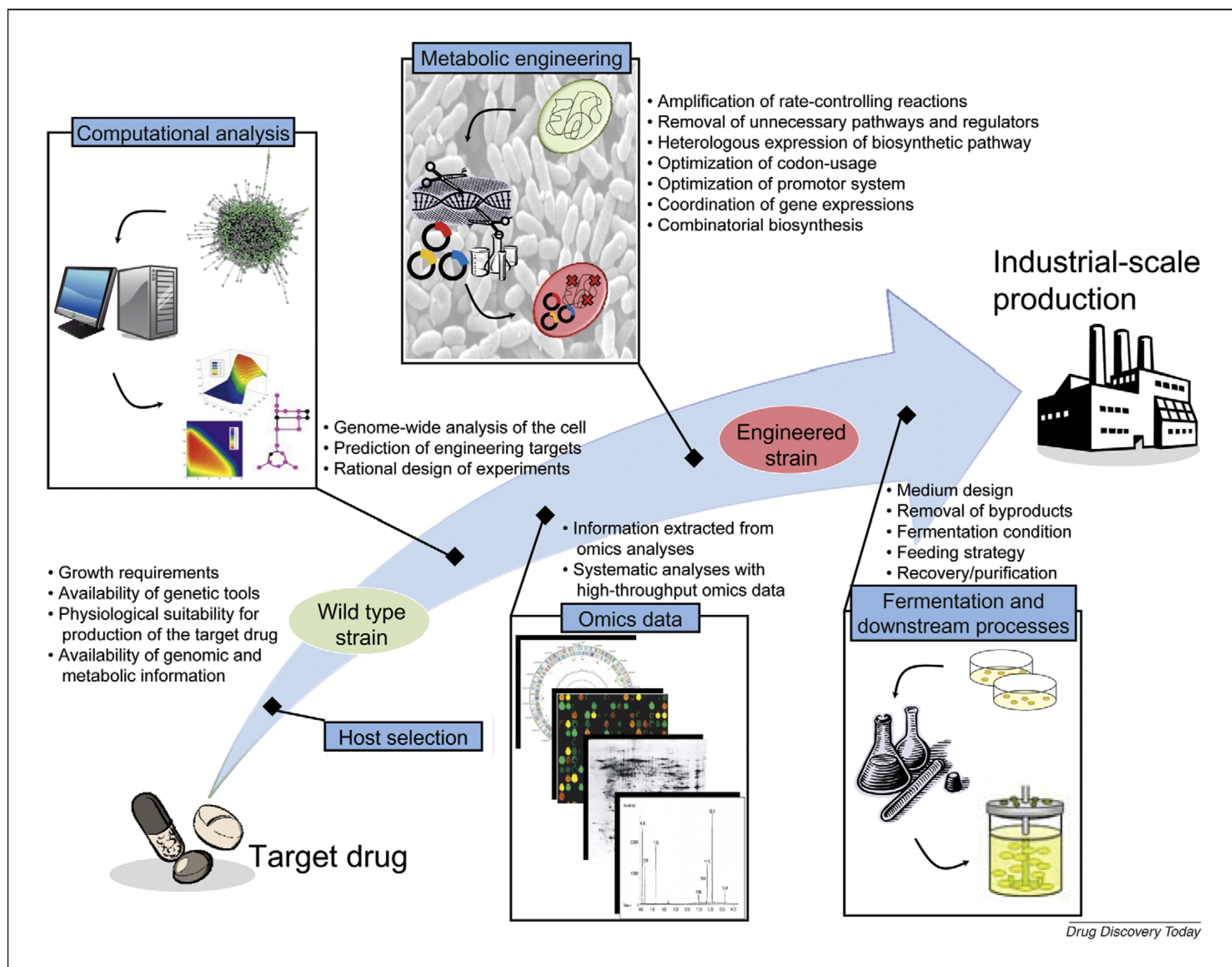

FIGURE 1

General strategy for the metabolic engineering of microorganisms for drug production. First consideration is to select which drug to produce. Then, the suitable host strain is selected by considering various factors including metabolic characteristics and capabilities to produce the drug of interest, culturability of the host strain, and availability of genetic engineering tools. Computational simulation and high-throughput omics analysis facilitate system-wide analysis of metabolic and cellular network, and prediction of the metabolic phenotype at the levels of transcript, protein, metabolite, and flux under various conditions. On the basis of the information, actual engineering is performed. Metabolic pathway design and optimization are performed by establishing new pathways, optimizing the existing pathways, and regulatory circuit engineering. Using the developed strain, fermentation and downstream processing are performed to produce the drug. On the basis of the fermentation performance and observations from recovery and purification processes, further metabolic engineering can be performed. The final strain developed in this cyclic optimization is used for the industrial production of the drug.

synthase (NCS) subsequently couples them to yield (*S*)-norlaudanosoline. Then, a series of enzymes derived from *Coptis japonica* are employed for the production of (*S*)-reticuline; (*S*)-norlaudanosoline is converted to (*S*)-3′-hydroxycoclaurine by norcoclaurine 6-*O*-methyltransferase (6OMT), (*S*)-3′-hydroxycoclaurine to (*S*)-3′-hydroxy-*N*-methylcoclaurine by coclaurine-*N*-methyltransferase (CNMT), and finally (*S*)-3′-hydroxy-*N*-methylcoclaurine to (*S*)-reticuline by 3′-hydroxy-*N*-methylcoclaurine-4′-*O*-methyltransferase (4′OMT). As a working example, two benzylisoquinoline alkaloids, magnoflorine and (*S*)-scoulerine, which can be used as antibacterial agents, were produced by co-culturing the engineered *E. coli* and *S. cerevisiae* cells using the precursor (*S*)-reticuline. The engineered *E. coli* was first grown in a medium containing dopamine to produce (*S*)-reticuline, and then *S. cerevisiae* expressing heterologous genes was added to the same medium to complete the production; different final products were produced, depending on the heterologous genes transformed into *S. cerevisiae*. Magnoflorine was produced when *S. cerevisiae* was engineered to harbor the genes encoding corytuberine synthase (P450 enzyme; CYP80G2) and coclaurine-*N*-methyltransferase (CNMT). When the berberine bridge enzyme (BBE) was expressed in *S. cerevisiae*, (*S*)-scoulerine was produced instead. As a result, various drug molecules can be produced by employing metabolically engineered *S. cerevisiae* with appropriate heterologous genes using the same precursor synthesized by engineered *E. coli*. This is a good example of what metabolic engineering can do for the design and production of drug precursors that are difficult to obtain otherwise.

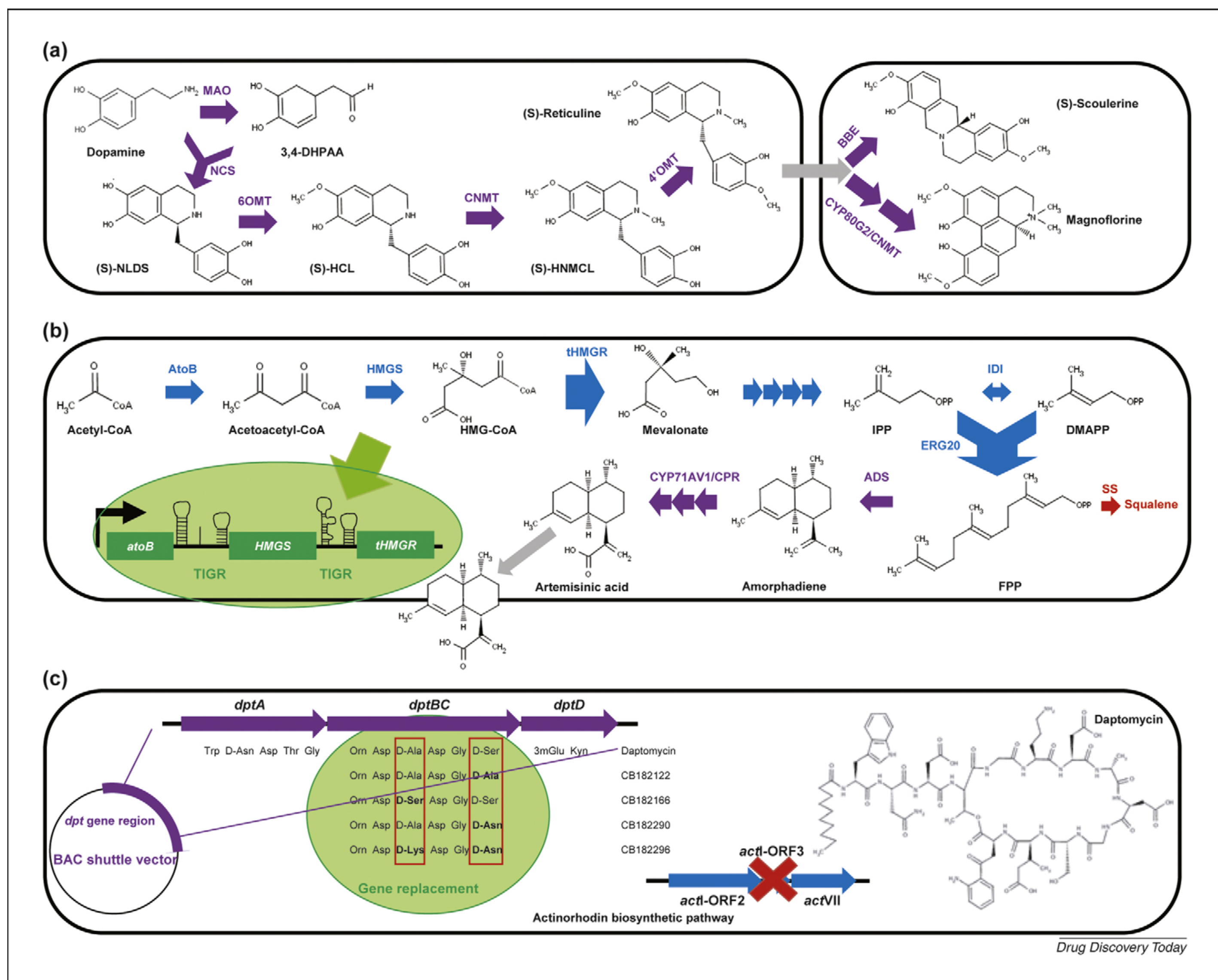

FIGURE 2

Metabolic engineering strategies employed for the production of benzylisoquinoline alkaloids, artemisinic acid, and daptomycin analogs. Purple arrows indicate expression of the heterologous genes. Blue arrows indicate homologous reactions in the host, and thick blue arrows indicate amplified reactions. Red arrow indicates downregulated gene, and gray arrows represent the transport of substances across the cell membrane. **(a)** Biosynthetic pathways for the production of (*S*)-reticuline and two benzylisoquinoline alkaloids, (*S*)-scoulerine and magnoflorine, constructed in *E. coli* (left) and *S. cerevisiae* (right), respectively. The alkaloids were produced by co-cultivation of these two microorganisms. **(b)** Biosynthetic pathways for artemisinic acid constructed in *S. cerevisiae*. Systematic engineering of each reaction step was performed as described in the text. Tunable intergenic regions (TIGRs) could be inserted between the biosynthetic genes in the FPP pathway and screened as performed in the engineered *E. coli*. Artemisinic acid produced was transported out of the cell, and bound on the cell surface. **(c)** Biosynthetic pathways for the synthesis of daptomycin. Initially, the 128 kb daptomycin gene cluster (*dpt*) was expressed in a heterologous host *S. lividans*. To further optimize detection and purification of daptomycin, the actinorhodin biosynthetic pathway (*act*) was inactivated, indicated by red 'X'. The culture medium was optimized by adding sufficient amount of phosphate. The expressed daptomycin gene cluster might be further optimized by combinatorial biosynthesis as done in *S. roseosporus*. Amino acids in the red box are those that were subject to directed mutagenesis by gene replacement. By doing so, various analogs of daptomycin could be created. Among them, four analogs that showed high potency are shown. Abbreviations are: 3,4-DHPAA, 3,4-dihydroxyphenylacetaldehyde; 4′OMT, 3′-hydroxy-*N*-methylcoclaurine-4′-*O*-methyltransferase; 6OMT, norcoclaurine 6-*O*-methyltransferase; ADS, amorphadiene synthase; AtoB: acetoacetyl-CoA thiolase; BAC, bacterial artificial chromosome; BBE, berberine bridge enzyme; cDNA of CYP71, CYP71AV1; CNMT, coclaurine-*N*-methyltransferase; CPR, cytochrome P450 oxidoreductase; CYP80G2, corytuberine synthase; DMAPP, dimethylallyl diphosphate; ERG20, FPP synthase; FPP, farnesyl pyrophosphate; HMGS, HMG-CoA synthase; IDI, IPP-DMAPP isomerase; IPP, isopentenyl diphosphate; MAO, monoamine oxidase; NCS, norcoclaurine synthase; SS, squalene synthase; tHMGR, truncated form of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; TIGR, tunable intergenic regions; (*S*)-NLDS, (*S*)-norlaudanosoline; (*S*)-HCL, (*S*)-3′-hydroxycoclaurine; (*S*)-HNMCL, (*S*)-3′-hydroxy-*N*-methylcoclaurine.

Expanding the roles of microorganisms

Table 1 summarizes recent achievements of genetic and metabolic engineering for the production of drugs and drug precursors. Besides diverse polyketides, various human health-related agents and plant-derived products have been produced by metabolically engineered microorganisms, which foreshadows that microorganisms, and in particular bacteria, might take over the roles of plant and mammalian cells in the near future [11,12]. For instance, bacteria are now able to perform glycosylation, an important post-translational modification to make protein drugs be functional, or even produce

full-length antibodies [11,12]. Small molecule drugs and drug precursors can be considered as even better target products that can be efficiently obtained by microbial metabolic engineering, as showcased in Table 1. Considering the rapid advances being made in microbial metabolic engineering, it is not too difficult to state that bacterial or microbial production system will replace the current production systems employing higher organisms.

General strategies for metabolic engineering of bacteria

Metabolic engineering strategies employed for the production of various chemicals are in essence valid for drug production as well (Figure 1). Basic criteria for the selection of host strain and genetic tools required for drug production are essentially the same. Optimization of metabolic pathways and networks is also the same requirement. In this section, we discuss key issues to be considered for successful metabolic engineering of microorganisms with respect to drug production, focusing on small molecule drugs.

Host strain

Host strain is a miniature chemical factory where drug of interest is produced, and, hence, is one of the most important factors to be considered. Several factors need to be considered when selecting a suitable host strain. One of the most important criterions is to examine the capability of host strain to perform the desired metabolic conversions; different microorganisms obviously possess different metabolic capabilities. *E. coli* has been the most favored strain for metabolic engineering, but one should be able to flexibly consider other microorganisms that might have better metabolic capabilities toward the production of desired products. For instance, although *E. coli* is an attractive host for pharmaceutical production because of its fast cell growth and availability of the best genetic engineering tools, it lacks enzymes necessary for polyketides assembly [20]. *Saccharopolyspora erythraea, Streptomyces fradiae, Streptomyces coelicolor, Streptomyces lividans*, and *Streptomyces venezualae* [21] are naturally better host microorganisms for the production of polyketide chemicals. Similarly, production of L-lysine and some other amino acids can still be best performed with corynebacterium even though metabolically engineered *E. coli* strains are performing well. In another example, succinic acid can be produced more efficiently by employing metabolically engineered rumen bacteria.

Availability of genetic engineering tools such as expression vectors, transformation protocol, and chromosomal gene knockout/integration system needs to be considered as these tools are essential for metabolic engineering. It is often necessary to perform overexpression of homologous and/or heterologous genes and knockout of those genes that are responsible for reduced product formation. Natural drug-producing microorganisms, particularly those producing antibiotics, might not have established gene manipulation systems, or are difficult to perform genetic engineering owing to their slow growth and fastidious growth requirements [21]. If these tools are not available, one can explore how difficult it is to develop them. Alternatively, one can select another host microorganism that has less ability to produce a desired product, but has much better gene manipulation system available.

The feasibility of cultivating the cells at various scales, including flask, small fermentor, pilot-scale fermentor, and industrial-scale fermentor, needs to be evaluated depending on the desired scale of operation. In most cases, the ability of host strain to grow on a simple medium using inexpensive carbon substrates needs to be checked because it is highly associated with cost-competitiveness in the bioprocesses, especially those for the production of bulk chemicals. Furthermore, the genetic and physiological backgrounds of host microorganisms need to be thoroughly examined as it is advantageous to choose the host that provides appropriate intracellular environment, generating sufficient amount of precursors or redox compounds required for coordinately regulating genes and successfully biosynthesizing functional drugs [22]. If this is not the case, more intensive metabolic engineering will be required to optimize the strain. Finally, availability of genomic and metabolic information will be beneficial when various components of the host are to be systematically exploited. However, it should be noted that whole genome sequencing is becoming less and less expensive, and thus determining the complete genome sequence of a host microorganism is not a formidable task anymore.

Gene manipulation

Once a host strain is decided, metabolic engineering strategies are developed. During this process, a series of gene manipulations are often required, which mainly consist of overexpression and knockout of genes and manipulation of regulatory circuits [23]. Overexpression and knockout of genes are performed to establish new metabolic pathways, to increase the metabolic fluxes toward the desired product, to decrease or shut down the undesirable metabolic fluxes, and to balance the metabolic fluxes for optimal product formation, which all contribute to enhancing product formation. However, there are complex regulatory mechanisms that control the flux redistribution. Thus, cellular and metabolic networks should be performed by analyzing the cellular system as a whole. One such recent example is metabolic engineering of *E. coli* for the efficient production of L-valine [4]. First, the biosynthetic pathways responsible for the formation of L-isoleucine, L-leucine, and pantothenate that compete with L-valine formation for the same precursor were blocked in the engineered *E. coli* strain, in which feedback inhibition and transcriptional attenuation controls that negatively affect L-valine production were removed by site-directed mutagenesis and chromosomal DNA replacement. Then, the first biosynthetic operon in the L-valine pathway was amplified. This initially engineered strain was further improved by systems-level analysis and engineering as will be described below. Another good example is the development of microbial strains for the heterologous production of benzylisoquinoline alkaloids [8] as described earlier.

In addition to systems-level analysis and engineering of metabolic and regulatory network, modification of expression levels of individual or multiple genes can be performed by engineering the promoters [24], codons [25], global transcriptional factors [26], and intergenic sequences [16,22]. All of these need to be performed in a coherent and systematic manner to develop superior production strains as observed in recent metabolic engineering achievements [22,27].

Use of high-throughput omics data

Large-scale genome-wide analysis, so called omics techniques, has enabled systems-level analysis of the cell, capturing the cell physiology in a holistic manner from several different angles [28,29]. The information extracted from omics analyses now serves to predict new engineering targets or re-design fermentation strategies that are difficult to be intuitively identified [30–33]. For example, transcriptome data that reveal gene expression levels at global-scale using DNA microarrays helped identifying a global regulator and exporter as amplification targets in the L-valine producing *E. coli* strain mentioned above [4]. Also, transcriptome data have been useful in analyzing the cell and re-designing the experiment for drug production as well. For instance, comparative transcriptome profiling was conducted for two *Streptomyces avermitilis* strains, one of them producing higher titer of avermectin, a commercial antiparasitic agent, and the other producing lower titer, in order to understand the genetic mechanisms that contribute to the overproduction of avermectin [32]. This consequently revealed several overexpressed genes in the superior strain, which could be used for reverse engineering of the strain for the overproduction of avermectin. In another case, transcriptome data and metabolite profiling were employed together to identify the cytotoxic causes of accumulated 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) in engineered *E. coli* that heterologously expresses the mevalonate pathway and overproduces isoprenoids, precursors of antimalarial drug artemisinin [33]. This analysis revealed that HMG-CoA inhibits the fatty acid metabolism of the host, and suggested a new fermentation strategy of providing palmitic and oleic acids in the medium. Proteome and metabolome data can be used similarly to identify new engineering targets as reviewed recently [33]. As these high-throughput omics analyses contribute to the systematic analysis of the cell, they are expected to be more frequently employed for drug production to identify and surmount the limitations of our biological knowledge and understanding.

Computational prediction of metabolic engineering targets

Although not often practiced yet, computational modeling and simulation are important tools for the metabolic engineering of microorganisms for drug production as they help characterize and engineer the cell at the systems-level [4,34]. Typical gene manipulations carried out during traditional metabolic engineering have been rather intuitive and limited to local components. Also, the consequences of gene manipulation are difficult to predict. Computational analysis of metabolic characteristics at genome-scale allows prediction of appropriate gene targets to be engineered by considering many different components constituting the biological system and their interactions, so that it suggests non-intuitive engineering strategies, and often provides more drastic results [35]. One of the popular computational methods for predicting gene targets to be engineered is constraints-based flux analysis, which begins with the genome-scale stoichiometric metabolic model that represents the mass balance information of metabolites in all cellular reactions [36]. On the basis of the assumption of pseudo-steady state, this system can be simulated by optimization techniques, typically linear programming, with various objective functions such as maximizing cell growth rate, maximizing the product formation rate, and minimizing the byproduct formation rate. This allows estimation of metabolic flux distribution in the whole cell [37]. The effects of gene knockout on whole cell metabolic flux distribution can be examined by constraining the flux value of the corresponding reaction to zero during the simulation. This simulation method has been proven to be a powerful tool for predicting metabolic engineering targets [4,5,38]. For instance, *in silico* knockout simulation using this method predicted three gene targets to be knocked out in the engineered L-valine-producing *E. coli*. By knocking out these three genes, the L-valine production yield could be increased to 148.7% [4]; these knockout targets are non-intuitive ones, thus suggesting the usefulness of genome-scale modeling and simulation in metabolic engineering. In addition, computational frameworks based on constraints-based flux analysis that guide addition of heterologous reactions [39] or upregulation and downregulation of homologous reactions have also been developed [40].

Besides application to identify gene targets for drug production, constraints-based flux analysis might also contribute to finding novel drug targets, an important facet of discovery and development of new drugs [41] and antimicrobials [42]. The latter is becoming more and more important as pathogens are constantly evolving to become resistant against various antimicrobials, and new superbugs are emerging [42]. To fight with these superbugs, novel antimicrobials that target different cellular functions of these microorganisms need to be developed [42]. Recently, a concept of metabolite essentiality using constraints-based flux analysis was reported, which is expected to provide a set of novel antimicrobial targets that systematically disrupt the pathogens' robustness [43]. Essential metabolites are defined as those that cause no cell growth upon their removal from the metabolic network. Because this phenomenon is equivalent to simultaneous deletion of outgoing reactions around the metabolite, metabolite essentiality simulation provides sets of reactions whose simultaneous deletion leads to cell death. As a result, metabolite essentiality analysis suggests multiple targets [43] that prevent cell growth upon their lack of function. Similar approaches can be taken to identify multiple targets for treating various human diseases. This example suggests that genome-scale computational analysis can be applied to early discovery phase of drug development, including both predicting drug targets and gene (protein) targets for the treatment of diseases.

Consideration of the overall bioprocess

One of the most important objectives of metabolic engineering is the efficient production of a desired product. It is essential to consider the whole bioprocess at an early stage of metabolic engineering. This is because metabolic engineering of microorganisms is typically performed in a process that involves test tube or flask cultivations. A supposedly superior strain developed in this way does not necessarily perform well in a large-scale fermentor, which might be used in actual production. Furthermore, one might encounter problems of byproducts contamination during the recovery and purification processes. One obvious metabolic engineering strategy is the removal of a cell's ability to form unnecessary byproducts as it greatly facilitates purification of the product; examples of microbial metabolic engineering for the homo-production of lactate [44], acetate [45], and ethanol [46] have been reported. Also, culture medium for microbial

fermentation should be carefully designed as its components would directly affect the recovery and purification processes and subsequently the cost of the overall bioprocess [47]; for example, depending on the product, a chemically defined medium or an inexpensive complex medium can be used. For the production of high price drug molecules, culture medium cost might not be an issue. It should be noted that several nutritional components should be added for auxotrophic or engineered mutants that do not grow or show undesirably retarded growth without supplementing them [4,33]. Furthermore, if the product is toxic, cells need to be engineered to improve their tolerance against the product. Transcription factor engineering is one such good example [26]. As an alternative, *in situ* separation that recovers the product directly from the bioreactor during fermentation, or a two-step fermentation process that runs two separate bioreactors for cell culture and product formation might be considered. Finally, it might be necessary to perform omics and computational analyses during the scale-up fermentation process to identify further metabolic engineering targets. Thus, systems metabolic engineering [48] needs to be performed to truly optimize the overall drug production process [49].

Examples of metabolic engineering for drug production

Several successful examples of applying metabolic engineering for the development of microbial strains producing drugs and drug precursors have recently been reported. Metabolic engineering of microbes for the production of artemisinic acid is one of the best examples that showcases the impact of intensive engineering of metabolic pathways on efficient drug production [7,27] (Figure 2b). Artemisinic acid is a precursor of artemisinin that is an effective drug against malaria-causing *Plasmodium* sp. Its biosynthesis in a microbe requires three steps: engineering mevalonate pathway using acetyl-CoA as a precursor to overproduce farnesyl pyrophosphate (FPP), conversion of FPP to amorphadiene by amorphadiene synthase (ADS), and finally oxidation of amorphadiene to artemisinic acid. First, the synthetic gene encoding ADS, designed on the basis of its cDNA isolated from *Artemisia annua* L (sweet wormwood), was cloned into *S. cerevisiae* [27]. In order to provide more FPP in this recombinant strain, a gene encoding the truncated form of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (tHMGR) that converts HMG-CoA to mevalonate in the FPP pathway and the *upc*2-1 gene, a global transcriptional factor of the sterol biosynthesis, were overexpressed, while squalene synthase that consumes FPP for the sterol biosynthesis was downregulated. Next step was to convert amorphadiene to artemisinic acid through a series of oxidations where comparative genomics played a crucial role in isolating relevant genes. Cytochrome P450 monooxygenase (P450) is known to catalyze this oxidation task in *A. annua*, and hence P450-expressed-sequence tags (ESTs) were first retrieved from two *Asteraceae* crops, sunflower and lettuce. Degenerate primers specific to the *Asteraceae* CYP71, the most abundant P450 subfamily, were then generated and used to isolate a gene encoding P450 from *A. annua*. Expression of its cDNA (CYP71AV1) along with its native redox partner cytochrome P450 oxidoreductase (CPR) in *S. cerevisiae* allowed successful production of artemisinic acid. During this work on the heterologous expression of P450, the importance of host selection was revealed because *E. coli* could not express the enzyme to a sufficient level; *E. coli* does not perform post-translational modification of enzymes and has more significant codon bias compared with *S. cerevisiae* [15]. This artemisinic acid-producing strain, however, showed a limited ability in supplying acetyl-CoA as a substrate for mevalonate pathway. Overexpression of acetaldehyde dehydrogenase and genetically engineered acetyl-CoA synthetase from *Salmonella enteric* (not subject to post-translational negative regulation) led to the highest level of amorphadiene production in *S. cerevisiae*, which was 22.5% greater than that achieved without these additional engineering [50]. Although not practiced in *S. cerevisiae*, application of tunable intergenic regions (TIGRs), which helps coordinating expression of genes by generating various intergenic structures between genes, would further increase the production level of amorphadiene and/or artemisinic acid [22] (Figure 2b). Before closing this successful story, it should be mentioned that cloning of plant genes for their successful expression in microorganisms might not be as easy as bacterial counterparts. This is because plant cell contains many similar genes and pathways devoted to secondary metabolites that are similar yet diversified, and plant genes are relatively scattered throughout the genome in contrast to bacterial genes that are in many cases clustered.

Another interesting example is the development of a strain for the production of daptomycin analog A21978C [16,51] (Figure 2c). Daptomycin is a non-ribosomal peptide produced in *Streptomyces roseosporus*, and is effective for treating skin infection caused by Gram-positive pathogens [16,51]. A21978C can be produced in the medium devoid of a lipid feed. The daptomycin biosynthesis genes were expressed in a heterologous host *S. lividans* by inserting them into the chromosome using the well-established genetic tools [51]. However, detection and purification of daptomycin were severely hampered owing to the production of other metabolites such as actinorhodins and several indefinite compounds belonging to the non-phosphorylated form of the calcium-dependent antibiotic (CDA) complex whose structures are similar to that of daptomycin. Thus, the genes responsible for the biosynthesis of actinorhodins (*act*) were inactivated by chromosomal inactivation, and the medium was optimized by adding sufficient amount of inorganic phosphate. The latter eventually phosphorylated the CDA complexes, which could readily be distinguished from daptomycin. As a consequence, the improved production of daptomycin in *S. lividans* compared to the original producer *S. roseosporus* could be realized.

Combinatorial biosynthesis of metabolites is a powerful method for generating various novel polyketides and non-ribosomal peptides with desirable drug functions [13,21]. Nguyen *et al*. applied the concept of combinatorial biosynthesis by engineering the biosynthetic gene cluster (*dpt*) of daptomycin, which ultimately alters the daptomycin cyclic peptide core and generates a novel lipopeptide library [16] (Figure 2c). The daptomycin biosynthetic gene cluster (*dpt*) contains the *dptA, dptBC*, and *dptD* genes, which encode daptomycin non-ribosomal peptide synthetase (NRPS) consisting of 13 amino acids (Figure 2c). Several gene replacement experiments were performed using λ-Red mediated recombination method. Two amino acid residues (D-Ala and D-Ser at position 8 and 11) in the *dptBC* gene were substituted with different amino acids. Likewise, 3mGlu (3-methyl-glutamic acid) at position 12 and kynurenine (Kyn) at position 13 of the *dptD* gene were

modified to several different amino acids. These various versions of two gene regions (*dptBC* and *dptD*) were finally combinatorially expressed in the cell, which yielded a variety of daptomycin analogs with modest antibacterial effects.

Many other examples of production of novel polyketides by metabolic engineering and combinatorial biosynthesis have also been reported. Modular reconstruction of polyketide synthase (PKS) modules has been a popular approach to generate a library of polyketide molecules [14]. In a recent example, unique sets of restriction sites were introduced into the flanking sites of each PKS modules to facilitate their interchanges; such unique flanking restriction sites allowed various combinatorial assembly of PKS modules [14,52]. The domains were assembled in *E. coli* in bimodular and trimodular combinations using two-plasmid system, each of which carries a functional module [14,52]. After establishing the combinatorial biosynthesis platform, additional engineering was performed to further improve polyketide production. For instance, the *eryK* gene encoding P450 hydroxylase and the *eryG* gene encoding S-adenosylmethionine-dependent *O*-methyltransferase were overexpressed at optimal ratios for the enhanced production of erythromycin A [53]. It should be noted here that simple random assembly of modules does not guarantee production of functional polyketides; besides selection of suitable host cells, kinetics, substrate specificities, stereochemical outcomes of the product, and even spatial relationships among domains should be carefully considered as well [21,52]. Furthermore, their efficient production will require strain optimization process by combining the approaches described above.

Perspectives

Metabolic engineering has enabled sophisticated engineering of various microorganisms for the efficient production of various metabolites, recently spurred by the high-throughput omics and genome-scale computational analyses [48]. What lies in the future? Metabolic engineering will play increasingly important role in developing new drugs and drug precursors, including secondary metabolites found in living organisms that have complex structure and chirality that are difficult to synthesize chemically. It will also allow creation of libraries of compounds that can be screened for new drugs. Once a new drug is discovered, one can follow the systems metabolic engineering strategy together with rational random approaches (such as transcription factor and promoter engineering) for its increased productivity; high-throughput omics techniques and genome-scale *in silico* simulations will be more actively employed to assess the characteristics of metabolic, regulatory, and signaling networks from various perspectives, which will enable system-wide prediction of which and how target genes, pathways, and even networks need to be engineered [48]. As our ability to analyze these heterogeneous data in an integrative manner advances, development of more robust strategy for metabolic engineering will be possible [54]. New genetic tools, such as application of tunable intergenic regions [22], and optimization of bioprocess variables, for instance medium composition [47], will further contribute to improving the productivity. Several successful examples of metabolic engineering for drug production have already appeared, and this trend will continue at an accelerating pace. It is expected that microbial metabolic engineering will become an essential platform for the development and production of drugs in the near future.

Reviews • POST SCREEN

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