ENGINEERING THE MICROBIAL PLATFORM FOR THE PRODUCTION OF BIOLOGICS AND SMALL-MOLECULE MEDICINES

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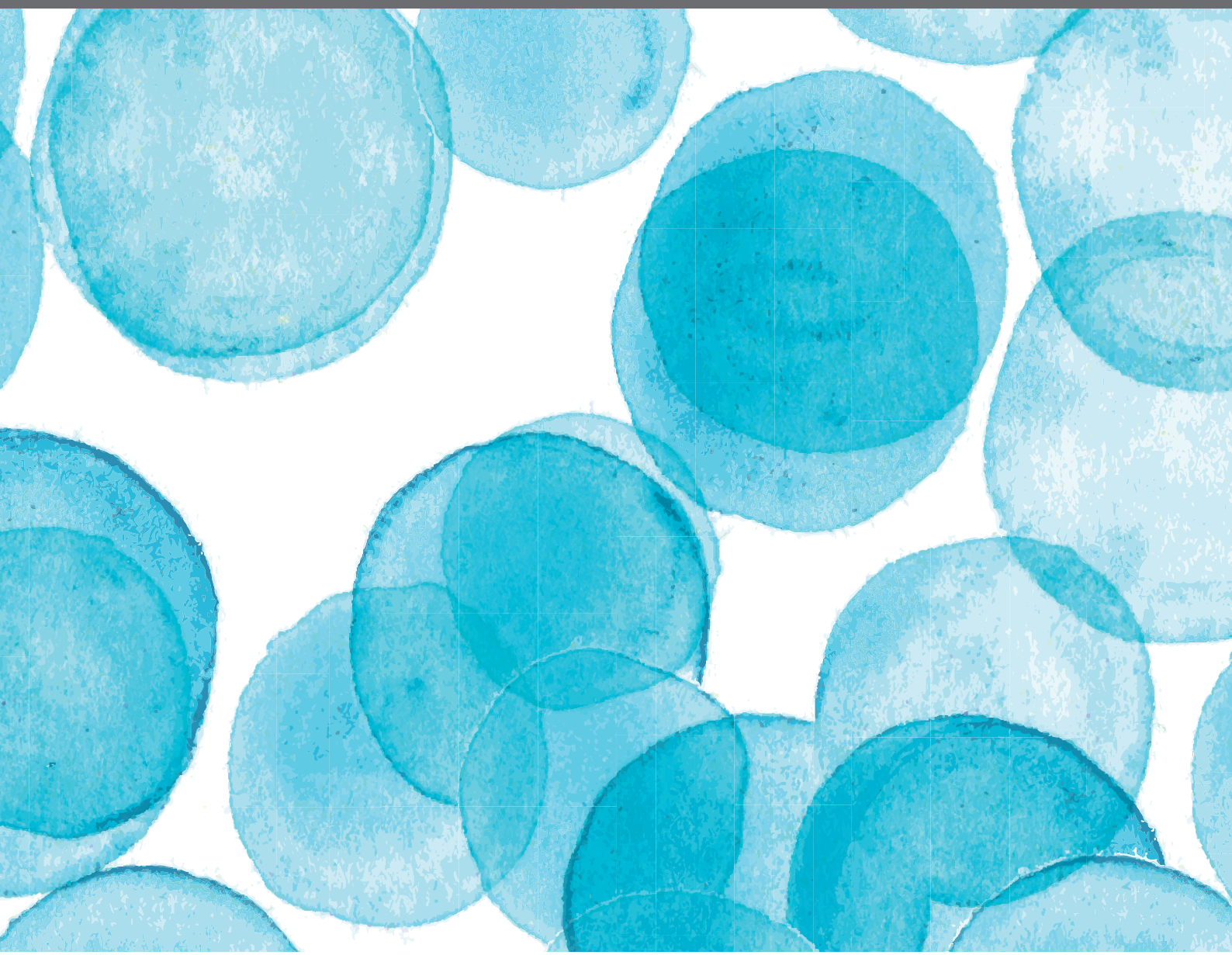

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ENGINEERING THE MICROBIAL PLATFORM FOR THE PRODUCTION OF BIOLOGICS AND SMALL-MOLECULE MEDICINES

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Editorial: Engineering the Microbial Platform for the Production of Biologics and Small-Molecule Medicines

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Editorial on the Research Topic

Engineering the Microbial Platform for the Production of Biologics and Small-Molecule Medicines

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Microorganisms are the prominent sources of valuable products ranging from large (e.g., proteins, carbohydrate polymers, nucleic acids, even cells) to small molecules (e.g., microbial metabolites, signaling molecules, growth factors, etc.). Most of these small molecules termed as "secondary metabolites (SM)s" are inessential to the producer for their growth and development. However, these SMs have significant applications in human and animal health (Demain, 2000; Bhan et al., 2013; Wang et al., 2016; Dhakal and Sohng, 2017). Besides, several biologics pharmaceutical ingredients extracted from animals, plants, and microorganisms such as antibodies, vaccines, receptor modulators or replacement/modulators of enzymes are applied for human welfare (Kinch, 2005; Lacana et al., 2007). The host microorganisms engineered for the production of such small molecular medicines or relatively complex biologics are termed as "microbial cell factories (MCF)." Recently, metabolic engineering approaches are developed for engineering of metabolism and biosynthetic pathways in these MCFs for better performance (Davy et al., 2017; Choi et al., 2018). The papers published in this Research Topic have attempted to explore the current state of the art of microbial engineering along with its diverse approaches.

Pham et al. have summarized the biological activities and applications of a variety of small molecular medicines and biologics. The manuscript has reviewed the diverse microbial systems used for the production of these biomolecules along with the versatile engineering strategies of such microbial platforms. Generally, each of the microbial strains can produce multiple compounds, but it can produce only subsets of these compounds under specific growth conditions. Therefore, variations in cultivation parameters can elicit the production and discovery of new SMs. For example, by changing cultivation parameters such as temperature, salinity, aeration, and even by altering the shape of the flasks, the production profile from a microbial platform can be altered (Bode et al., 2002). Pan et al. have provided comprehensive information regarding the exploration of structural diversity of microbe secondary metabolites using one strain many compounds (OSMAC) approach. They have presented the role of variation in medium, cultivation conditions, use of epigenetic modifiers, and co-cultivation in the discovery of novel secondary metabolites from diverse microbial sources utilizing OSMAC approach (Pan et al.).

Escherichia coli is reported as the most common cell factory for the production of both small molecules and biologics. The clear understanding of its physiological and genetic characteristics, fast-growth even in minimal salts medium, and availability

of easy genetic manipulation techniques has established it as first-choice production host (Liu et al., 2015). Also, systems metabolic engineering approaches that combine knowledge of systems biology, synthetic biology, and evolutionary engineering into the traditional metabolic engineering, has facilitated the development of *E. coli* as a robust production host for heterologous expression of small molecules and complex biologics (Choi et al., 2019). So, different metabolic engineering approaches utilizing *E. coli* as microbial platform have been presented in this Research Topic. Wang et al. have reviewed different aspects of terpenoid production using *E. coli* including the metabolic engineering and genome engineering approaches. Li et al. utilized product resistance and targeted metabolic engineering for the production of equol in *E. coli*. Similarly, the combination of product tolerance, evolution engineering, and modular co-culture was utilized for the production of pinene (Niu et al.). The metabolic engineering approach utilizing gene over-expression cassette for enhanced production of nucleotide diphosphate (NDP)-sugars was utilized for generating the salicylate glucoside and other glycosylated variants (Qi et al.). Similarly, the gene-silencing approach was employed for enriching the titer of 3′-phosphoadenosine-5′-phosphosulfate (PAPS), which is donor substrate for sulfation of natural product (NP) precursors. Hence, by inhibiting the degradation of PAPS mediated by repression of PAPS reductase (*cysH*) and optimization of different sulfate donors significantly enhanced the production titer of naringenin-7-sulfate (Chu et al.). Ribosomally synthesized and post-translationally modified peptides (RiPPs) are special class of NPs with diverse structures and bioactivities, and thus possess a complex biosynthetic mechanism. Different aspects for heterologous expression of RiPPs in *E. coli* have been reviewed by Zhang et al..

Due to the presence of endotoxins in products obtained from Gram negative bacteria as *E. coli*, some of the non-lethal Gram-positive bacteria including the native producer strains such as actinobacteria or heterologous hosts [generally recognized as safe (GRAS)] such as *Bacillus* and *Corynebacterium* are used as excellent cell factories in industries. Actinobacteria are characterized as the most prominent producers of thousands of bioactive molecules, particularly small molecular medicines such as commercially available antibiotics and anticancer-drugs (Dhakal et al., 2017; Rangseekaew and Pathom-aree, 2019). In some cases, NPs from these actinomycetes are cryptic or not produced in a significant amount. Thus, precise metabolic engineering can be employed in a native host or genetically tractable alternative heterologous hosts for significant production. Li et al. performed whole genome sequencing of the producer strain, analyzed the genome data by computational tools and isolated nocardamine utilizing genome mining of *Streptomyces atratus* SCSIOZH16. Peng et al. used *S. lividans* as platform organism and optimized the host for higher heterologous expression of foreign biosynthetic gene cluster (BCG) by modulation by a number of global positive and negative regulatory genes, and genes encoding drug efflux pumps. Further the optimized strain was used for production of NPs of diverse nature such as actinorhodin, murayaquinone, hybrubins, piericidin A1, dehydrorabelomycin, and actinomycin D. Generally, the production of SMs in *Streptomyces* is controlled by a complex regulatory network that involves pathway-specific, pleiotropic, and global regulators, which tune the expression level of biosynthetic genes in response to a variation in diverse physiological and environmental conditions (van Wezel and McDowall, 2011). Hence, the engineering of such regulation cascades by activators and repressors have significant role in determining the productivity of target molecules. Yu et al. identified AdpAch, as a bidirectional pleiotropic regulator of natamycin biosynthesis in *S. chattanoogensis* L10. Subsequently, the production titer of natamycin was enhanced by mutating the AdpAch-binding sites, that had an inhibitory effect. Recently, the application of precise genetic engineering based on clustered regularly interspaced short palindromic repeats (CRISPER) and its associated protein (Cas9) has enabled the multiplexed genome engineering of actinomycetes including *Streptomyces*. Tao et al. have reviewed the application of CRISPR/Cas9 based genome editing in *Streptomyces* for discovery, characterization, and production of NPs. The recent advances in heterologous expression of RiPPs in *Streptomyces* have been presented by Zhang et al..

Bacillus species has an ability to adapt to varying environmental conditions and capacity for high production yield (Pham et al.), hence they are crucial industrial microorganisms. Further, the application of recent advances in metabolic engineering, enzyme/pathway engineering along with the synthetic biological tools have contributed to ameliorate the production titer from these microorganisms. Yang et al. utilized enzyme engineering of homogentisate dioxygenase for production of enhanced production of melanin. Similarly, a metabolic engineering approach was utilized for enhanced heterologous production of 2-deoxy-scyllo-inosose in *Bacillus subtilis*. Unlike *E.coli* and *Bacillus*, *Corynebacterium* has significant ability to utilize a variety of carbon sources (Heider and Wendisch, 2015). *C. glutanicum* is established as a major industrial producer of proteins, including biologics and enzymes as well as utilized in the production of diverse secondary metabolites as carotenoids, terpenes, and flavonoids. Lee and Kim have reviewed different crucial aspects of recombinant protein expression systems in *C. glutanicum* and its applications.

Fungi is the second largest kingdom of microorganism after bacteria. They are established as a promising source of bioactive natural products containing unique chemical compounds against various diseases (Singh et al., 2019). Ever since *Penicillium notatum* was identified as a source of penicillin, there has been immense interest in the exploration of the potential of fungal species for their capacity to produce versatile NPs with biotechnological and pharmaceutical applications. Guzmán-Chávez et al. have summarized on the engineering aspects of the *P. chrysogenum* for establishing it as a sustainable cell factory for NPs. They have provided the comprehensive summary about the basic biosynthetic logic of such NPs and various rational strategies for activation of biosynthetic gene clusters by optimizing culture parameters or targeted genetic engineering Guzmán-Chávez et al. In addition to the bacteria and fungi, the yeast strain such as *Saccharomyces cerevisiae* is successfully employed for the production of both bulk and fine chemicals (Kavščček et al., 2015). The different aspects of

biosynthesis and prospects of metabolic engineering for the production of terpenoids in *S. cerevisiae* are summarized by Wang et al..

Taken together, all these papers illustrate the applicability of engineering of microbial platforms for the production of small molecular medicines to complex biologics. However, in case of all of these microbial cell factories (native, engineered, or heterologous) the industrial scale titer, yield, and productivity is generally difficult to achieve. The major constraint is unavailability of abundant information about their metabolic behavior, unavailability of appropriate genetic engineering tools, or complication in redesigning appropriate flux balance for diverting primary metabolites to target molecules (Bhan et al., 2013). Recently the application of large-scale genome sequencing, gene expression profiling, *in silico* metabolic modeling and simulation, and enzyme/pathway engineering has eased the rational approaches for metabolic engineering. Particularly, the traditional approach of single strain/pathway specific "try and test" approach is replaced by the application of systems metabolic engineering approach that utilizes integration of strain selection/development, pathway design/engineering, and enzyme selection/engineering for efficient production of target molecules. In addition, the application of tools for generating artificial genetic circuits/metabolic pathways incorporating efficient promoters, RBS, terminators, etc, or multiplexed genome engineering utilizing CRISPR/ Cas9 for gene knock-in/knock out, or activation/repression has advanced the engineering approaches of these MCFs to the next level. In future, it can be expected that it can be feasible to generate the super host with minimized genome and enriched metabolic pathway centered on particular class of molecules. Such super hosts can be engineered by introducing the synthetic genome to attain the designers' strain for specific target. The burgeoning development in both genetic studies as well as computational approaches such as artificial intelligence (AI) has great prospects for simulating the connection between the genomics and metabolomics to generate the intelligence in these super hosts, so that they can sense the environment condition, and respond rationally.

AUTHOR CONTRIBUTIONS

DD wrote the manuscript. E-SK and MK revised and corrected the manuscript. The final draft of the manuscript was finalized and approved for publication by all the authors.

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Bidirectional Regulation of $AdpA_{ch}$ in Controlling the Expression of *scnRI* and *scnRII* in the Natamycin Biosynthesis of *Streptomyces chattanoogensis* L10

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AdpA, an AraC/XylS family protein, had been proved as a key regulator for secondary metabolism and morphological differentiation in *Streptomyces griseus*. Here, we identify $AdpA_{ch}$, an ortholog of AdpA, as a "higher level" pleiotropic regulator of natamycin biosynthesis with bidirectional regulatory ability in *Streptomyces chattanoogensis* L10. DNase I footprinting revealed six $AdpA_{ch}$-binding sites in the *scnRI–scnRII* intergenic region. Further analysis using the *xylE* reporter gene fused to the *scnRI–scnRII* intergenic region of mutated binding sites demonstrated that the expression of *scnRI* and *scnRII* was under the control of $AdpA_{ch}$. $AdpA_{ch}$ showed a bi-stable regulatory ability where it firstly binds to the Site C and Site D to activate the transcription of the two pathway-specific genes, *scnRI* and *scnRII*, and then binds to other sites where it acts as an inhibitor. When Site A and Site F were mutated *in vivo*, the production of natamycin was increased by 21% and 25%, respectively. These findings indicated an autoregulatory mechanism where $AdpA_{ch}$ serves as a master switch with bidirectional regulation for natamycin biosynthesis.

Keywords: bidirectional regulation, AdpA, natamycin biosynthesis, *Streptomyces chattanoogensis* L10, pathway-specific gene

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INTRODUCTION

The secondary metabolic process in *Streptomyces* is regulated by a complex regulatory network involving pathway-specific, pleiotropic, and global regulators which respond to a variety of physiological and environmental condition alterations (van Wezel and McDowall, 2011; Liu et al., 2013). The best characterized is the A-factor regulatory cascade in which AdpA is the most important transcriptional factor for the secondary metabolism (Horinouchi, 2002; Ohnishi et al., 2005). In early culture stages, the transcription of *adpA* in *Streptomyces griseus* is repressed by ArpA, the receptor protein for A-factor (Onaka and Horinouchi, 1997). When A-factor reaches a critical concentration, it binds to ArpA and confers the conformational change of ArpA (Ohnishi et al., 1999). This results in dissociation of ArpA from the *adpA* promoter, in turn switching on the expression of *adpA* (Ohnishi et al., 1999). The induced AdpA then activates the transcription of

various genes related to secondary metabolism such as *strR*, the pathway-specific regulatory genes for streptomycin in *S. griseus* (Retzlaff and Distler, 1995; Tomono et al., 2005).

AdpA is a member of the AraC/XylS family proteins (Gallegos et al., 1997). It has been suggested to form a dimer through the N-terminal portion which belong to the ThiJ/PfpI/DJ-1 family (Yamazaki et al., 2004; Ohnishi et al., 2005). To date, a number of AdpA orthologs have been described as having essential roles in the secondary metabolism in many *Streptomyces* species, such as *Streptomyces lividans* (Guyet et al., 2013), *Streptomyces coelicolor* A3(2) (Takano et al., 2001; Nguyen et al., 2003), *Streptomyces ansochromogenes* (Pan et al., 2009), *Streptomyces avermitilis* (Komatsu et al., 2010), *Streptomyces hygroscopicus* 5008 (Tan et al., 2015), and *Streptomyces clavuligerus* (López-García et al., 2010).

Typically, AdpA is regarded as an activator for downstream regulated genes, except itself which is proved to be negatively auto-regulated by binding to its own promoter region (Kato et al., 2005b; Hara et al., 2009). The molecular mechanism of transcriptional activation begins as a dimer of AdpA binds to the target sites with consensus sequences which then recruit RNA polymerase to the promoter for transcriptional initiation (Yamazaki et al., 2004; Kato et al., 2005a). For different target genes, AdpA showed a different number of binding sites in the promoter regions. For example, there are two AdpA-binding sites in the promoter of *strR* (Tomono et al., 2005), whereas there are three AdpA-binding sites for regulation of *ssgA* (Yamazaki et al., 2003a). However, the precise regulation mechanism how the AdpA binds to multiple sites to activate transcription has not been experimentally determined. Based on the importance of AdpA in the biosynthesis of the secondary metabolism, it is necessary to elucidate details of its regulatory mechanisms.

Natamycin, an antifungal polyene macrolide antibiotic, is synthesized by a type I polyketide synthase gene cluster. Previous analysis of the gene cluster of natamycin in *Streptomyces chattanoogensis* L10 revealed the existence of 17 open-reading frames, including two pathway-specific genes, *scnRI* and *scnRII* (Du et al., 2011a). These two genes showed high sequence identity to *pimR* and *pimM* of *Streptomyces natalensis*, respectively (Antón et al., 2007; Santos-Aberturas et al., 2012). Gene disruption of *scnRI* resulted in a large decrease in the expression of biosynthetic genes, indicating its role as a pivotal activator for the biosynthesis of natamycin (Du et al., 2011a). *scnRII*, adjacent but divergently transcribed transcriptional regulatory genes, was shown to act as a second positive regulator for natamycin production (Du et al., 2009). We also had proved that $AdpA_{ch}$ controls the production of natamycin, but the detailed relationship among $AdpA_{ch}$, ScnRI, and ScnRII had not been well characterized (Du et al., 2011a).

Here, we reveal the sophisticated regulatory characteristics of $AdpA_{ch}$ in the natamycin biosynthesis of *S. chattanoogensis* L10. $AdpA_{ch}$ acts as a "higher level" pleiotropic regulator for transcription of the two divergently transcribed pathway-specific genes, *scnRI* and *scnRII*. In this regulatory process, $AdpA_{ch}$ shows a bi-stable regulatory ability, where it firstly acts as an activator, then a repressor. Moreover, natamycin production was enhanced by mutating the $AdpA_{ch}$-binding sites which had an inhibitory effect. This work not only advances the understanding of detailed regulatory mechanism of AdpA, but also provides a potential target for the enhancement of other antibiotic production levels by manipulating the regulatory network.

RESULTS

$AdpA_{ch}$ Identified as a "Higher Level" Pleiotropic Regulator for Natamycin Biosynthesis

In our previous study, the biosynthetic gene cluster of natamycin has been cloned and characterized in *S. chattanoogensis* L10. Within this there are two divergently transcribed genes, *scnRI* and *scnRII*, encoding proteins that resemble pathway-specific regulators (Du et al., 2009, 2011a). Although the functions of these two regulators have been well characterized, an important question remains as to whether there are multiple levels of control in the biosynthesis of natamycin. Based on our previous study that $AdpA_{ch}$ affected the transcription of these two pathway-specific genes (Du et al., 2011a), we speculated that $AdpA_{ch}$ may act as a "higher level" pleiotropic regulator for regulating the natamycin biosynthesis.

To test this hypothesis, electrophoretic mobility shift assays (EMSAs) were applied. As shown in **Figure 1**, retardation was readily detected upon the addition of 50 pM $AdpA_{ch}$ with the probe RI–RII, while the addition of 50- to 100-fold excess of unlabeled specific PCR product reduced the proportion of the labeled promoter-containing fragment (**Figure 1**). These data clearly demonstrate that $AdpA_{ch}$ could specifically bind to the *scnRI–scnRII* intergenic region and could control the expression of these two pathway-specific genes.

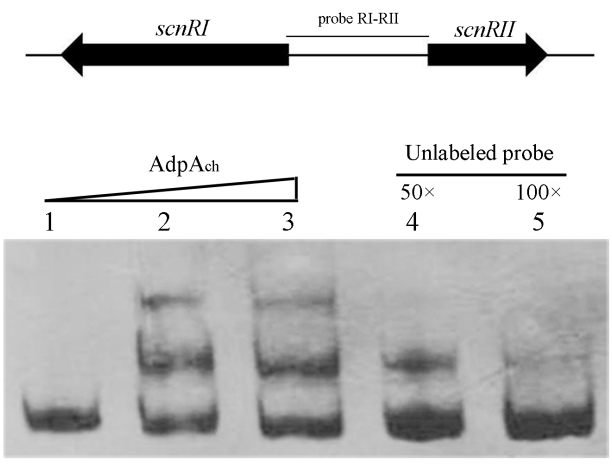

FIGURE 1 | $AdpA_{ch}$ binds to the DNA sequence of the intergenic promoter region between *scnRI* and *scnRII*. Lanes 1–3, DNA probe with $AdpA_{ch}$ protein 0, 50, and 100 pM, respectively. Lanes 4 and 5, 50- and 100-fold excess of unlabeled specific PCR product was added into binding reactions.

DNase I Footprinting Assay Reveals Six $AdpA_{ch}$-Binding Sites in the *scnRI–scnRII* Intergenic Region

To identify the exact DNA sequences that $AdpA_{ch}$ protected in the *scnRI–scnRII* intergenic region, DNase I footprinting assays, in absence or presence of purified recombinant $AdpA_{ch}$, were performed. In our previous studies, we had determined the transcription start site (TSS) of the two pathway-specific genes, *scnRI* and *scnRII* (Du et al., 2011a). As seen in **Figure 2A**, at a lower $AdpA_{ch}$ protein concentration of 100 pM, the DNA strands of the *scnRI–scnRII* intergenic region showed two protected regions, Site C and Site D, extending from positions −69 to −44 and −106 to −74 relative to the TSS of *scnRI*. When increasing the protein concentration to 500 pM, another four protected regions (Sites A, B, E, and F) were observed. With respect to the *scnRI* TSS, the $AdpA_{ch}$-binding Site A locates at positions +8 to +54, Site B at positions −20 to +2, Site E at positions −161 to −114, and Site F at positions −283 to −259 (**Figure 2B**). The six $AdpA_{ch}$-binding sites were spread over the *scnRI–scnRII* intergenic region. Notably, Site A was located downstream of the *scnRI* TSS, while Site B overlapped the −10 region of the *scnRI* promoter. Site F was located downstream of the *scnRII* TSS, and Site E overlapped the −35 region of the *scnRII* promoter. This data suggest that $AdpA_{ch}$ might have a negative regulatory ability for the expression of these two pathway-specific genes. Additionally, the results from the DNase I footprinting assay also reveal that $AdpA_{ch}$ may have higher affinity to Site C and Site D than to the others.

The Consensus $AdpA_{ch}$-Binding Sequence in the $AdpA_{ch}$-Binding Sites

The orthologs of $AdpA_{ch}$ identified in *S. griseus* and *S. coelicolor* have been reported to have the consensus binding sequence, 5-TGGCSNGWWY-3 (S: G or C; W: A or T; Y: T or C; N: any nucleotide) (Yamazaki et al., 2004). After alignment of these six protected regions, we also found that there were highly conserved $AdpA_{ch}$-binding sequences in each binding site (**Figure 3A**). To further study the roles of these consensus sequences in the $AdpA_{ch}$-binding ability, EMSAs were carried out using the probes containing either the sequences of wild-type (wt) binding sites or the mutated sites (**Figure 3A**). As shown in **Figure 3B**, no binding shift was detected for the mutated sites A–F when compared with their corresponding wt targets. Taken together, these data demonstrated that $AdpA_{ch}$ indeed

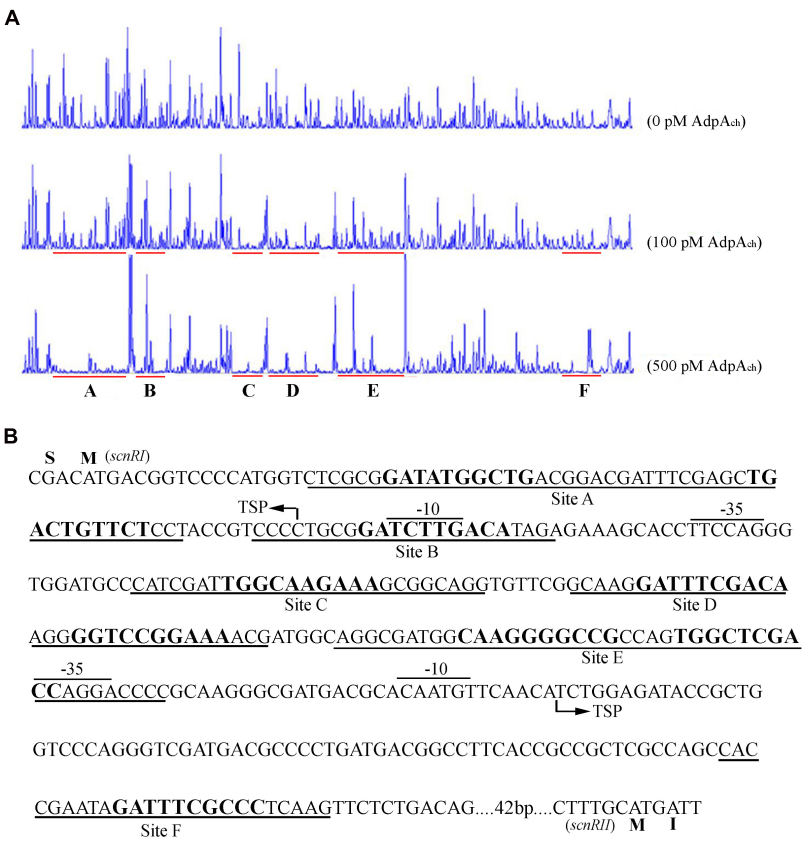

FIGURE 2 | DNase I footprinting assay for determination of the $AdpA_{ch}$-binding sites. **(A)** A 5′-FAM-labeled probe p*RI-RII* was used in the DNase I footprinting assay with 0, 100, and 500 pM purified $AdpA_{ch}$, respectively. The protected regions are underlined. **(B)** Nucleotide sequences of the *scnRI–scnRII* intergenic region showing the predicted $AdpA_{ch}$-binding sites. The TSS is marked by a bent arrow, the $AdpA_{ch}$-binding sites are underlined, and the −10 and −35 regions are overlined.

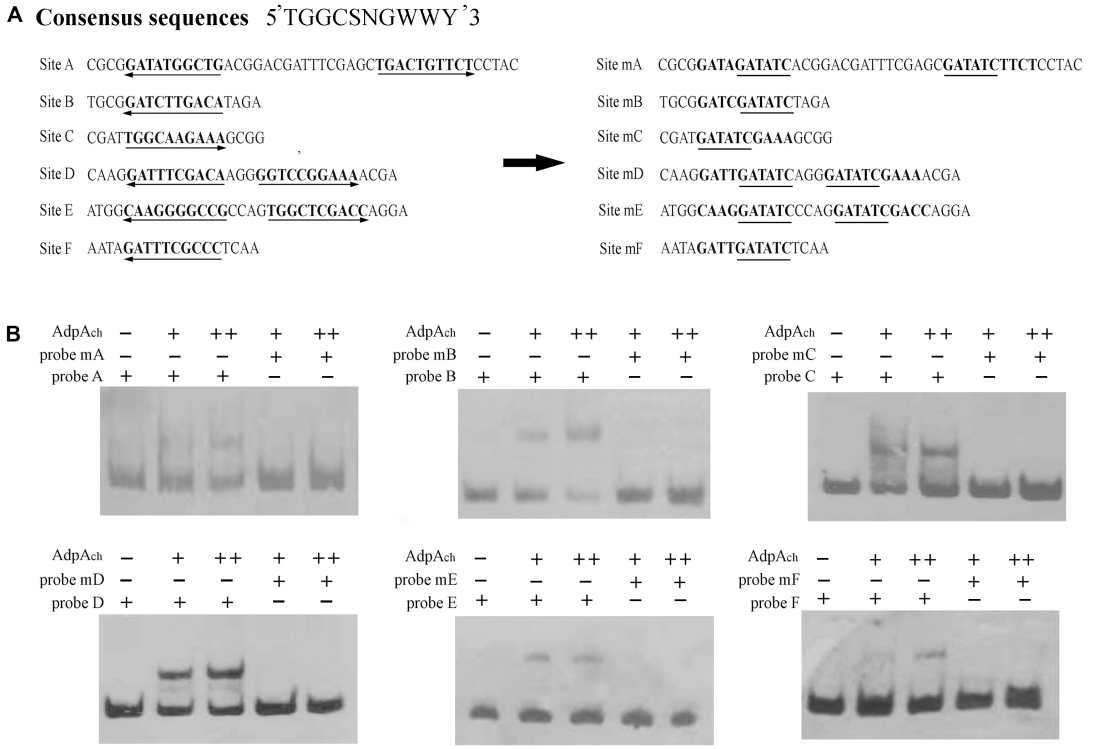

FIGURE 3 | Mutational analysis of the $AdpA_{ch}$-binding sites. **(A)** Mutations introduced in the six putative $AdpA_{ch}$-binding sites. The predicted $AdpA_{ch}$-binding consensus sequences are in bold, and these consensus sequences are changed with an *EcoRI* site indicated with underlines. **(B)** EMSAs for determination of $AdpA_{ch}$ binding to mutated sequences. Probes A–F contained the fragment of Sites A–F as shown in **A**, respectively. Probes mA–mF contained the fragment of corresponding mutated sites. The amounts of $AdpA_{ch}$ protein used were 50 and 100 pM.

has six binding sites in the *scnRI–scnRII* intergenic region and the consensus sequence is essential for the binding activity of $AdpA_{ch}$.

$AdpA_{ch}$ Has Differing Affinities for Different Binding Sites

In the DNase I footprinting analysis, Site C and Site D were occupied with a lower concentration of $AdpA_{ch}$ than the other sites. This suggests that there may be affinity differences for $AdpA_{ch}$ between the six binding sites. To test this possibility, competitive EMSAs with 50- to 100-fold excess of unlabeled fragments of six $AdpA_{ch}$-binding sites were used to compete with each labeled fragment. As shown in **Figure 4A**, 100-fold excess of unlabeled S_B' (Site B) and S_F' (Site F) could not completely abolish $AdpA_{ch}$ complex formation with the labeled probe S_A (Site A). However, the same amount of unlabeled S_C' (Site C), S_D' (Site D), and S_E' (Site E) outcompeted the labeled probe S_A. This result indicated that $AdpA_{ch}$ binds to Site A more tightly than Site B and Site F, but less tightly than Site C, Site D, and Site E. Following this way, we could conclude that Site B has less affinity for $AdpA_{ch}$ than others, except for Site F (**Figure 4B**), which was the weakest affinity among the six binding sites (**Figure 4F**), and Site D was the strongest affinity of these six sites (**Figure 4D**). The affinity of Site E for $AdpA_{ch}$ was between that of Site C and Site A (**Figures 4A,C,E**). Therefore, we determined the affinity of $AdpA_{ch}$ to different binding sites in the following order: Site D > Site C > Site E > Site A > Site B > Site F.

Promoter-Probe Assays of the $AdpA_{ch}$-Binding Sites in the *scnRI–scnRII* Intergenic Region

The binding sites of $AdpA_{ch}$ in the *scnRI–scnRII* intergenic region were adjacent to either the *scnRI* or the *scnRII* start codon. This raised the possibility that this intergenic region might harbor a bidirectional promoter allowing $AdpA_{ch}$ to regulate transcriptions of the divergently transcribed flanking genes, *scnRI* and *scnRII* (**Figure 2B**). To investigate the promoter activities of the two pathway-specific genes with each of the $AdpA_{ch}$-binding sites, we used the promoter-probe plasmid pIJ8601 carrying the *xylE* gene, encoding catechol 2,3-dioxygenase, as the reporter. As shown in **Figure 5A**, the transcriptional profiles of *scnRI* were severely decreased when the $AdpA_{ch}$-binding Site C and Site D were mutated. Conversely, its transcriptional activity was increased when Site A and Site B were mutated and remained almost unchanged when Site E and Site F were mutated. For the promoter activity of *scnRII*, we did not detect any consistent differences when Sites A, B, and C were mutated, but mutation in the Sites D and E resulted in a large decreases of up to 70 and 40%, respectively, compared to those of the wt. The mutation in Site F resulted in a statistically significant increase

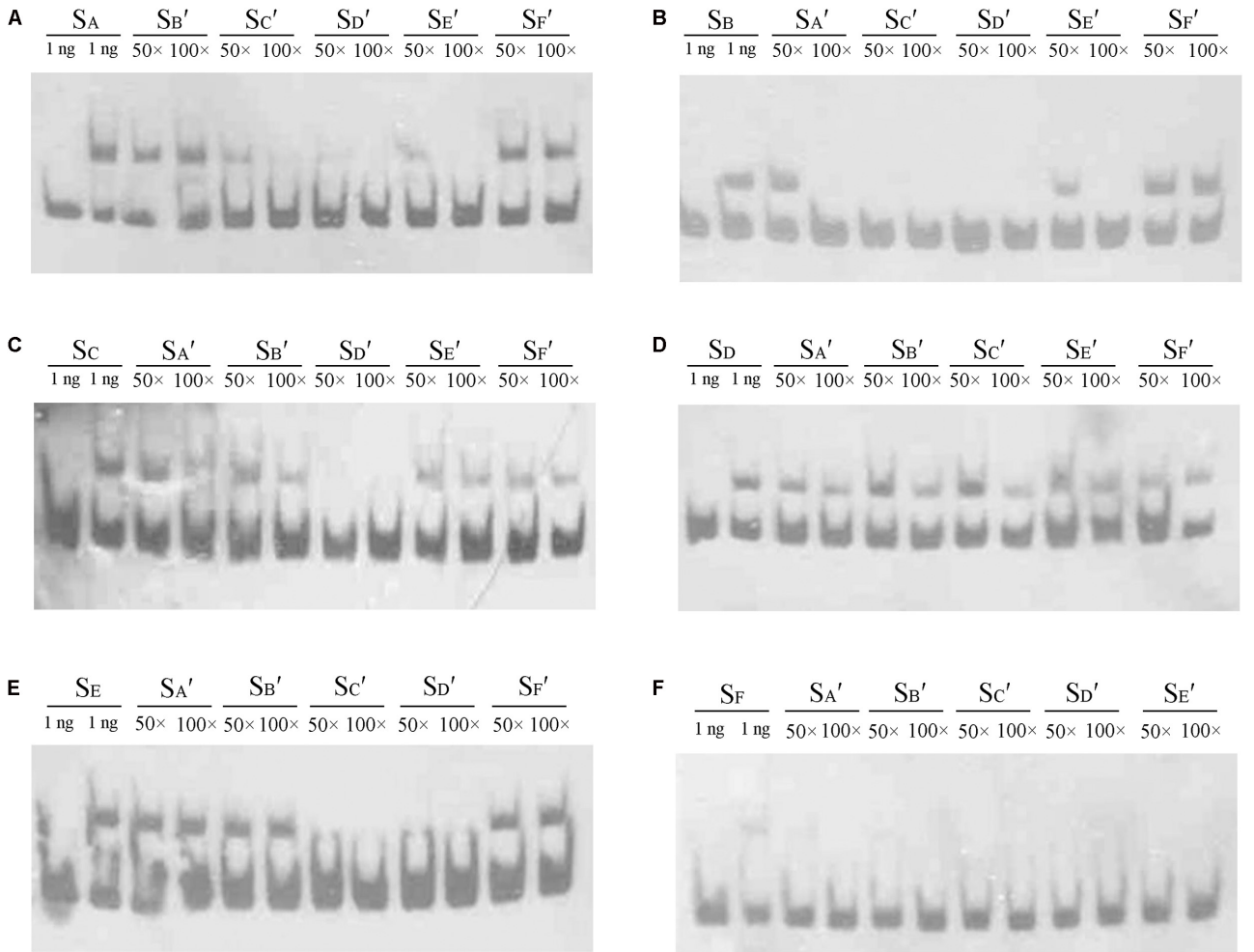

FIGURE 4 | Comparison of the relative affinity of $AdpA_{ch}$ with different binding sites. Labeled probes S_A, S_B, S_C, S_D, S_E, and S_F contained the fragment of Sites A–F as shown in **Figure 3A**, respectively. Probes $S_{A'}$, $S_{B'}$, $S_{C'}$, $S_{D'}$, $S_{E'}$, and $S_{F'}$ also contained the fragment of Sites A–F as shown in **Figure 3A**, respectively, but they are unlabeled. The amount of $AdpA_{ch}$ protein used was 100 pM.

(**Figure 5B**). These findings indicated that expressions of *scnRI* and *scnRII* are both under the control of $AdpA_{ch}$, which has a completely different regulatory ability (activation or inhibition) when binding to different binding sites.

Effect of Mutated $AdpA_{ch}$-Binding Sites on Natamycin Production *in Vivo*

There have been some reports where effects upon DNA-binding sites were found *in vitro* that failed to be exhibited *in vivo*. In order to test this possibility and reveal the function of the six $AdpA_{ch}$-binding sites in natamycin biosynthesis *in vivo*, a series of mutants were constructed as described in Experimental procedures. As shown in **Figure 6A**, compared to the WT strain, the level of natamycin production in the R-mA (mutation in Site A) and R-mF (mutation in Site F) had increased by 21 and 25%, respectively. However, the constructed strains of R-mC (mutation in Site C), R-mD (mutation in Site D), and R-mE (mutation in Site E) showed up to 31, 42 and 15% reductions, respectively. The natamycin production of R-mB (mutation in Site B) mutant exhibited almost no change. This finding indicated that the $AdpA_{ch}$-binding Sites A and F play negative roles for natamycin biosynthesis, while the functions of the Sites C, D, and E were positive. Quantitative real-time PCR (qRT-PCR) analysis showed that the promoting effect of site mutation on natamycin production was due to alteration of the pathway-specific genes at the transcriptional level (**Figure 6B**).

DISCUSSION

Streptomyces spp. have developed complicated mechanisms to adapt to altered circumstances (Santos-Beneit et al., 2009; Yu et al., 2012). Among these mechanisms, the multiple levels of regulation in controlling the expression of the genes responsible for the formation of the secondary metabolism are drawing increased attention. In this study, we focused on the regulatory network of natamycin biosynthesis in *S. chattanoogensis* L10,

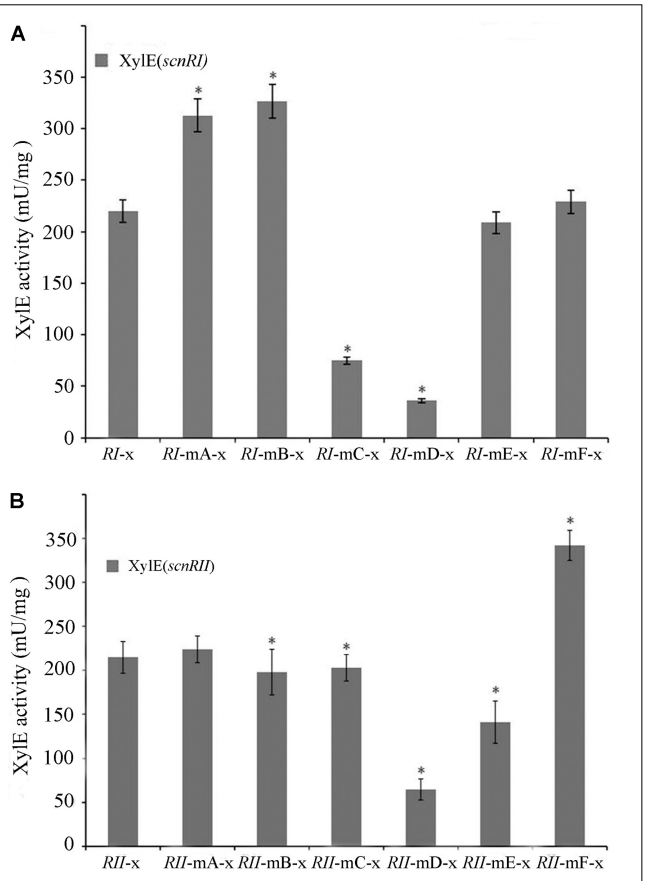

FIGURE 5 | Promoter activities of *scnRI* **(A)** and *scnRII* **(B)** with the effect of mutations in the $AdpA_{ch}$-binding sites. The strains were grown in YEME medium for 24 h, and catechol dioxygenase activity was calculated as the change of catechol quantity (mmol) per minute. Error bars correspond to the standard error of the mean of four culture replicates. * Indicates significant differences between promoter mutants and promoter wt ($P < 0.05$).

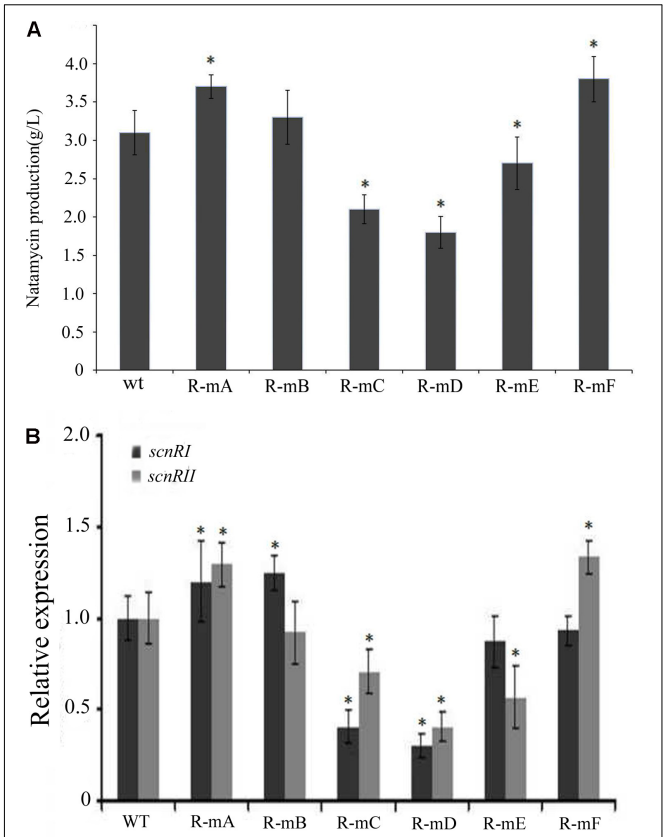

FIGURE 6 | (A) The effect of mutated $AdpA_{ch}$-binding sites on the natamycin production *in vivo*. The strains were grown in YEME medium for 96 h. Vertical error bars correspond to the standard error of the mean of four replicated cultures. **(B)** Real-time RT-PCR analysis of the *scnRI* and *scnRII* transcript in the wt strain and mutated $AdpA_{ch}$-binding sites strain. The expression level of *scnRI and scnRII* is presented relative to the wt sample from 24 h, which was arbitrarily assigned a value of 1. The transcription of *hrdB* was assayed as an internal control. Error bars were calculated by measuring the standard deviation among three replicates of each sample. * Indicates significant differences between $AdpA_{ch}$-binding site mutants and wt ($P < 0.05$).

an industrial strain for natamycin production. In our previous study, we determined that gamma-butyrolactones (GBLs) serve as quorum-sensing signaling molecules for activating natamycin production in *S. chattanoogensis* L10 (Du et al., 2011b), and ScnRII acts as a positive regulator by directly binding to the promoters of natamycin biosynthetic genes (Du et al., 2009) where ScnRI acts as a positive regulator for the transcription of *scnRII* (Du et al., 2011a). However, the deletion of *scnRI* did not result in a complete halt of the transcription of *scnRII* (our unpublished data). This is quite different from the function of PimR in *S. natalensis* where the deletion of *pimR* almost completely destroys the transcription of *pimM* (Antón et al., 2004; Santos-Aberturas et al., 2012). As the regulation of antibiotic biosynthesis involves numerous transcription factors (McKenzie and Nodwell, 2007; van Wezel and McDowall, 2011), participation of other regulator(s) is possible, in the regulation of *scnRII*.

With $AdpA_{ch}$ being able to regulate the expression of both of the pathway-specific genes, *scnRI* and *scnRII*, it provides a possible explanation that there is a coordinate regulation in controlling expression of *scnRII* by $AdpA_{ch}$ and ScnRI. This regulatory pattern may occur in following steps. Firstly, $AdpA_{ch}$ binds to the *scnRI–scnRII* intergenic region and activates both transcription of *scnRI* and *scnRII*. Then ScnRI also binds to the *scnRI–scnRII* intergenic region which, in turn, promotes the transcriptional level of *scnRII*. However, these two genes were not completely controlled by $AdpA_{ch}$. Trace expression of *scnRI* was observed in the $adpA_{ch}$ mutant, and then ScnRI would promote the transcription of *scnRII* (Du et al., 2011a). Notably, a certain amount of $AdpA_{ch}$ is required for binding to the *scnRI–scnRII* intergenic region (∼50 pM). This is why we did not detect the shifted band with low concentration $AdpA_{ch}$ (∼1 pM) in the binding reaction of our previous study (Du et al., 2011a).

In most cases, AdpA acts as an activator for the target genes, except for itself where it exhibits an autorepression (Kato et al., 2005b). In this study, we concluded from promoter-probe assays *in vivo* that $AdpA_{ch}$ could not only regulate both

pathway-specific genes, but also displayed completely opposite regulatory abilities in control of them. The $AdpA_{ch}$-binding Site C and Site D were involved in activating the transcription of *scnRI*, while $AdpA_{ch}$ binding to Sites A and B resulted in repression. For the promoter activity of *scnRII*, mutation in the Site C and Site D resulted in a decrease of transcriptional profiles, while a mutation in the Site F led to a statistically significant increase. A similar phenotype was observed in *S. ansochromogenes* where transcription of *sanG* decreased when Site I and Site V were mutated but increased when other three AdpA-L-binding sites were mutated (Pan et al., 2009). However, when combinations of binding site mutations were carried out, the promoter activities were not in accordance with our predictions. For example, mutations in both Sites E and F reduced the transcriptional level of *scnRII* (data not shown). Based on the short distances between the $AdpA_{ch}$-binding sites which are spread over the *scnRI–scnRII* intergenic region, there may be complicated interactions between different $AdpA_{ch}$ dimmers to explain this.

With further analysis using competitive gel shift assays, we could conclude that $AdpA_{ch}$ binds to Sites A–F with the following affinities: Site D > Site C > Site E > Site A > Site B > Site F (**Figure 4**). These data are consistent with the footprinting assay where the regions of Site C and Site D were previously protected at a lower $AdpA_{ch}$ protein concentration (**Figure 2A**). This gives a hint that the regulatory ability of $AdpA_{ch}$ may occur in a growth phase-dependent manner. In the early stage, $AdpA_{ch}$ firstly binds to the Site C and D to recruit RNA polymerase to the promoter and initiates the transcription of *scnRI* and *scnRII*. This in turn triggers natamycin production (**Figure 7**). When $AdpA_{ch}$ is accumulated to a certain critical level, it will bind to other binding sites located near the TSS. A DNA loop may be formed via the interaction between different $AdpA_{ch}$ dimers, thus preventing RNA polymerase from access to the promoter of the pathway-specific genes (**Figure 7**). Reduced transcription of the pathway-specific genes will result in a low rate of natamycin production.

The discovery of this bidirectional regulation of $AdpA_{ch}$ in the control of natamycin biosynthesis reveals an artful adaptive mechanism in microbial cells. Microorganisms produce molecules with antibiotic activity and expel them into the environment, presumably enhancing their ability to compete with their neighbors (Berdy, 2005; Hopwood, 2007). However, most of these molecules are toxic to the producer (Mak et al., 2014; Moody, 2014). Mechanisms must exist to ensure that antibiotic production reaches a reasonable level. The proposed model of $AdpA_{ch}$ in **Figure 7** may provide a fresh mechanistic insight into how *S. chattanoogensis* controls the production level of natamycin via $AdpA_{ch}$. However, further work will be needed to prove the proposed model and the detailed mechanism of how $AdpA_{ch}$ responds to the signal of natamycin. In all, the complicated regulatory network involving $AdpA_{ch}$, ScnRI, and ScnRII helps advance our understanding of the molecular regulation mechanisms of antibiotic biosynthesis and provides an effective strategy to help improve yields in industrial strains.

MATERIALS AND METHODS

Media, Plasmids, Strains, and Growth Conditions

All plasmids and bacterial strains used in this study are listed in **Table 1**. General techniques for the manipulation of nucleic acids and bacterial growth were carried out according to the standard protocols as previously described (Kieser et al., 2000). *Escherichia coli* DH5α was the general cloning host. Vectors used were pSET152, pIJ8660, pTA2. *S. chattanoogensis* L10 strains were grown at 28°C on YMG agar for sporulation and at 30°C

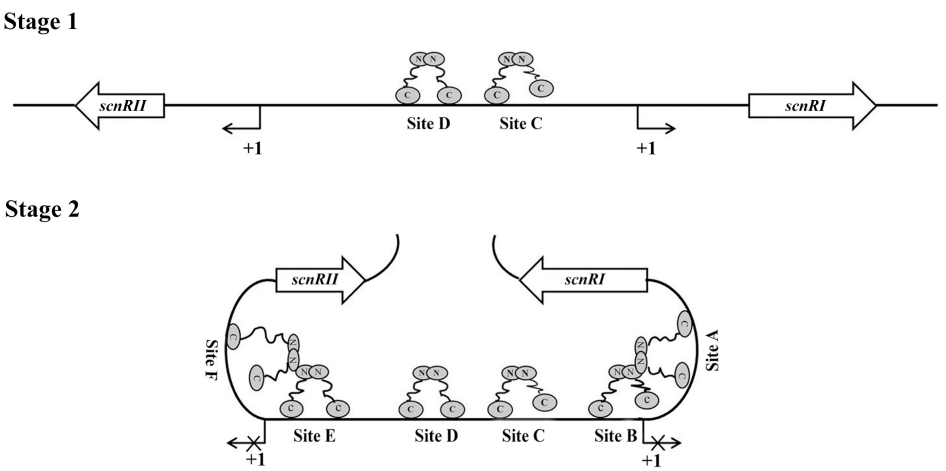

FIGURE 7 | Proposed model of $AdpA_{ch}$ regulation for *scnRI* and *scnRII* transcription. The $AdpA_{ch}$-binding sites are spread over the *scnRI–scnRII* intergenic region, and the presumptive manner of $AdpA_{ch}$-binding forms a dimmer. Stage 1: $AdpA_{ch}$ binds to the Site C and Site D when the concentration of $AdpA_{ch}$ is low in the early growth stage. Stage 2: When the concentration $AdpA_{ch}$ reaches a high level, $AdpA_{ch}$ binds to Site A, Site B, Site E, and Site F, most of which located downstream of the −35 sequences of the corresponding genes. A DNA loop may be formed via interaction between different $AdpA_{ch}$ dimmers, thus preventing RNA polymerase from access to the promoter.

TABLE 1 | Bacterial strains and plasmids used in this work.

Strains/plasmids	Characteristics	Reference
Strains		
E. coli TG1	General cloning host	Novagen
E. coli ET12567/pUZ8002	Methylation-deficient *E. coli* for conjugation with the helper plasmid	Macneil and Klapko, 1987
E. coli BL21 (DE3)	A host for protein expression	Novagen
E. coli BW25113/pIJ790	Strain used for PCR-targeted mutagenesis	Gust et al., 2003
Wt	*S. chattanoogensis* L10 wt; natamycin producer	Du et al., 2009
RI-x	wt with pIJ8601-*pRI*	This study
RI-mA-x	wt with pIJ8601-*pRI-mA*	This study
RI-mB-x	wt with pIJ8601-*pRI-mB*	This study
RI-mC-x	wt with pIJ8601-*pRI-mC*	This study
RI-mD-x	wt with pIJ8601-*pRI-mD*	This study
RI-mE-x	wt with pIJ8601-*pRI-mE*	This study
RI-mF-x	wt with pIJ8601-*pRI-mF*	This study
RII-x	wt with pIJ8601-*pRII*	This study
RII-mA-x	wt with pIJ8601-*pRII-mA*	This study
RII-mB-x	wt with pIJ8601-*pRII-mB*	This study
RII-mC-x	wt with pIJ8601-*pRII-mC*	This study
RII-mD-x	wt with pIJ8601-*pRII-mD*	This study
RII-mE-x	wt with pIJ8601-*pRII-mE*	This study
RII-mF-x	wt with pIJ8601-*pRII-mF*	This study
R-mA	wt with mutation in Site A	This study
R-mB	wt with mutation in Site B	This study
R-mC	wt with mutation in Site C	This study
R-mD	wt with mutation in Site D	This study
R-mE	wt with mutation in Site E	This study
R-mF	wt with mutation in Site F	This study
Plasmids		
pTA2 vector	General cloning vector	TOYOBO
p-*RI-RII*	pTA2 containing the fragment of the *scnRI–scnRII* intergenic region	This study
pIJ8601	Streptomyces integrative shuttle vector with *xylE* reporter gene	This study
pIJ8601-*pRI*	pIJ8601 with the promoter of *scnRI*	This study
pIJ8601-*pRI-mA*	pIJ8601-*pRI* with mutation in Site A	This study
pIJ8601-*pRI-mB*	pIJ8601-*pRI* with mutation in Site B	This study
pIJ8601-*pRI-mC*	pIJ8601-*pRI* with mutation in Site C	This study
pIJ8601-*pRI-mD*	pIJ8601-*pRI* with mutation in Site D	This study
pIJ8601-*pRI-mE*	pIJ8601-*pRI* with mutation in Site E	This study
pIJ8601-*pRI-mF*	pIJ8601-*pRI* with mutation in Site F	This study
pIJ8601-*pRII*	pIJ8601 with the promoter of *scnRII*	This study
pIJ8601-*pRII-mA*	pIJ8601-*pRII* with mutation in Site A	This study
pIJ8601-*pRII-mB*	pIJ8601-*pRII* with mutation in Site B	This study
pIJ8601-*pRII-mC*	pIJ8601-*pRII* with mutation in Site C	This study
pIJ8601-*pRII-mD*	pIJ8601-*pRII* with mutation in Site D	This study
pIJ8601-*pRII-mE*	pIJ8601-*pRII* with mutation in Site E	This study
pIJ8601-*pRII-mF*	pIJ8601-*pRII* with mutation in Site F	This study

in YEME medium (3 g/l yeast extract, 3 g/l malt extract, 5 g/l tryptone, 10 g/l glucose) for natamycin production.

Electrophoretic Mobility-Shift Assays (EMSAs)

His-AdpA$_{ch}$, histidine-tagged protein was purified from the soluble fractions of *E. coli* BL21 (DE3) harboring the plasmids pET32a-*adpA*$_{ch}$, as previously described (Du et al., 2011a). The Bradford reagent (Bio-Rad) was used to determine the protein concentration. For probe preparation, all primers used in this study are listed in Supplementary Table S1. The EMSA DNA probe RI–RII (517 bp) spanning the entire *scnRI–scnRII* intergenic region was amplified by PCR using primer pair RI–RII-F and RI–RII-R. The PCR product was then cloned into a pTA2-vector (TOYOBO) to generate the plasmid pT-*RI–RII*. The biotin-labeled probe RI–RII was made with 5′-biotin-labeled M13 universal primer pair using pT-*RI–RII* as a template by PCR

amplification. The probes A (295 bp), B (281 bp), C (294 bp), D (282 bp), E (288 bp), F (284 bp), mA (295 bp), mB (281 bp), mC (294 bp), mD (282 bp), mE (288 bp), and mF (284 bp) were prepared following the above-mentioned method. In the EMSAs assay, 1 ng of the probe was incubated with varying quantities of $AdpA_{ch}$, at 25°C for 30 min in the buffer (20 mM Tris, pH 7.5, 5% glycerol, 0.01% BSA, 50 $\mu g\ ml^{-1}$ sheared sperm DNA). For the competition assay, 100 times of excessive un-labeled probes and non-specific DNA were added to the reaction buffer, respectively. Reactions were displayed on 5% acrylamide gels for separation in 0.5× TBE buffer. EMSA gels were then electro-blotted onto the nylon membrane and UV-fixed by UV crosslinker. Labeled DNA was detected with streptavidin-HRP and BeyoECL plus (Beyotime, China) as described by the manufacturer.

DNase I Footprinting Assay

DNase I footprinting assay was performed as previously described (Mao et al., 2009). Firstly, $AdpA_{ch}$ protein was ultra-filtered with YM-10 (Millipore) for 10 kD cut-off and eluted in 20 mM Tris buffer, pH 7.5. Then, FAM-labeled probe was amplified using 5′-(6-FAM)-labeled M13 universal primers from plasmid pT-*RI-RII*, followed by gel recovery. About 50 ng of fluorescently labeled probe was added to the reaction mixture to a final volume of 50 μl. After binding of the $AdpA_{ch}$ protein to 5′-(6-FAM)-labeled probe (30°c, 30 min), 0.01 U of DNase I (Promega) was added for 1 min at 30°C, followed with equal volume of 100 mM EDTA to stop the reactions and extracted by phenol/chloroform. After precipitation with 40 μg of glycogen, 0.75 M ammonium acetate (NH_4Ac), and ethanol, the digested DNA mixture was loaded into ABI 3130 DNA sequencer with Liz-500 DNA marker (MCLAB). DNA sequencing ladder was prepared according to Thermo Sequenase Dye Primer Manual Cycle Sequencing Kit (USB).

Alterations of the Consensus Sequence for $AdpA_{ch}$-Binding Sites

The consensus sequence of $AdpA_{ch}$-binding sites A–F was replaced by the sequence of EcoRV restriction sequence sites using overlapping primers (Supplementary Table S1). The PCR product was then cloned into a pTA2-vector (TOYOBO). The resulted plasmids were used as template for PCR to amplify mutated probes using 5′-biotin-labeled M13 universal primers, and the binding ability was measured by EMSAs.

Construction and Analysis of Transcriptional Fusions to the *xylE* Reporter Gene

For *xylE* fusions, the *xylE* gene was PCR amplified with the primers *xylE*-F and *xylE*-R. This fragment was digested with *Nde*I and *Not*I, and introduced into the likewise-digested pIJ8660 (Sun et al., 1999) to construct pIJ8601. To probe *scnRIp* and *scnRIIp* activities with the mutation of $AdpA_{ch}$-binding sites, the wt and mutated promoter regions were amplified by PCR using upstream primers carrying a BamHI site listed in Supplementary Table S1. These promoter fragments were cloned into BamHI-cut pIJ8601 and transferred by conjugation into *S. chattanoogensis* L10. Plasmid-containing strains were grown on YEME medium for 24 h. Cell pellets from 1 ml culture samples were kept on ice and measured immediately. Assays of catechol 2,3-dioxygenase were performed as previously described (Kieser et al., 2000).

Mutational Analysis of the $AdpA_{ch}$-Binding Sites on Natamycin Biosynthesis

The 1.8 kb DNA fragment containing the sequence of *scnRI–scnRII* intergenic region was amplified by PCR using primers scnRI-F and scnRII-R. The resulted 1.8 kb sequence was used as template to amplify the DNA fragment for construction of mutated $AdpA_{ch}$-binding sites *in vivo* using overlapping primers (Supplementary Table S1), then PCR product was purified and ligated into pKC1139. The resulting plasmids containing DNA fragment of mutated sites was conjugated by *E. coli* ET12567/pUZ8002 into *S. chattanoogensis* L10. The mutants were selected by replica plating for apramycin-sensitive colonies and they were used as template for PCR with primer pairs RI-RII-F and RI-RII-R. The amplified sequences were digested with EcoRV to confirm the mutants.

Determination of Natamycin Production by HPLC Analysis

Natamycin production was confirmed by HPLC analysis with the Agilent 1100 HPLC system. HC-C_{18} column (5 μm, 4.6 by 250 mm) was used with UV detector set at 303 nm. Mobile phase and gradient elution process were as described previously (Du et al., 2009).

AUTHOR CONTRIBUTIONS

PY, Q-TB, and Y-LT performed the experiments. X-MM assisted with the primary data analysis. Y-QL supervised the project and revised the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00316/full#supplementary-material

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To Construct an Engineered (*S*)-Equol Resistant *E. coli* for *in Vitro* (*S*)-Equol Production

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(*S*)-equol is one of the major metabolites of daidzein that is produced by human and animal gut bacteria. Most of the physiological functions of soybean isoflavones, such as anti-oxidative activity, anti-cancer activity, and cardiovascular protection have been ascribed to (*S*)-equol. However, only 30–50% people contain this kind of equol-producing bacteria, and therefore are able to convert daidzein to (*S*)-equol. Administration of (*S*)-equol may be more beneficial than soybean isoflavones. The aim of this study was to construct an engineered (*S*)-equol resistant *Escherichia coli* to enhance (*S*)-equol production *in vitro*. First, transposon mutagenesis libraries were constructed and screened to isolate the (*S*)-equol resistant mutant *E. coli* strain BL21 (*ydiS*) in order to overcome the inhibitory effects of (*S*)-equol on bacterial growth. Bacterial full genome scan sequencing and *in vitro* overexpression results revealed that the *ydiS* gene was responsible for this resistance. Second, the (*S*)-equol-producing genes L-*dznr*, L-*ddrc*, L-*dhdr*, and L-*thdr* of *Lactococcus* strain 20–92 were synthesized and cloned into compatible vectors, pETDuet-1 and pCDFDuet-1. These plasmids were subsequently transformed into BL21 (DE3) and its mutant BL21 (*ydiS*). Both engineered BL21 (DE3) and BL21 (*ydiS*) could use daidzein as substrate to produce (*S*)-equol under both anaerobic and aerobic conditions. As expected, engineered BL21 (*ydiS*) had faster growth rates than BL21 (DE3) when supplemented with high concentrations of (*S*)-equol. The yield and the daidzein utilization ratio were higher for engineered BL21 (*ydiS*). Interestingly, engineered BL21 (*ydiS*) was able to convert daidzein to (*S*)-equol efficiently under aerobic conditions, providing a convenient method for (*S*)-equol production *in vitro*. In addition, a two-step method was developed to produce (*S*)-equol using daidzin as substrate.

Keywords: (S)-equol production, (*S*)-equol resistance, soybean isoflavone, transposon mutagenesis, *ydiS* gene

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INTRODUCTION

Soy isoflavones have multiple health benefits due to their anti-carcinogenic, anti-oxidant, and anti-atherosclerotic properties (Xiao et al., 2017). These chemicals also interact with the estrogen receptor, enabling them to act as weak to moderate phytoestrogens (Nielsen and Williamson, 2007). Interestingly, a variety of studies have suggested that the clinical effectiveness of isoflavones might

be due to their metabolites (Setchell et al., 2002; Sarkar and Li, 2003; Cobb et al., 2006; Cooke, 2006; Jackman et al., 2007). In 2002, Setchell et al. (2002) proposed the "Equol Hypothesis," which posits that daidzein is converted to (*S*)-equol by gut bacteria in certain individuals, and that it is the equol that accounts for the noted health benefits of soy isoflavones.

Equol (7-hydroxy-3-[4-hydroxyphenyl]-chroman) was first isolated from equine urine in 1932 (Marrian and Haslewood, 1932) and was also identified 50 years later in human urine as a metabolite of soy isoflavones (Axelson et al., 1982). Equol is not present in soybeans, but it is produced naturally in the human gut by daidzein, a major isoflavone predominantly found in soybean, by intestinal bacteria (Setchell and Clerici, 2010). Equol exhibits stronger anti-oxidant and estrogenic activities than daidzein (Hwang et al., 2003; Kinjo et al., 2004; Turner et al., 2004; Rufer and Kulling, 2006) and has been demonstrated to act as vasorelaxant (Jackman et al., 2007), along with having anti-inflammatory properties (Blay et al., 2010), which have both been observed previously in soy isoflavones (Chacko et al., 2005; Hall et al., 2008). However, only 30–50% of the human population can produce equol (Low et al., 2005; Ozasa et al., 2005; Setchell and Cole, 2006; Hall et al., 2007). This suggests that health effects of functional foods supplemented with (*S*)-equol could be more beneficial than daidzein. Several studies have demonstrated that a diet supplemented with natural (*S*)-equol alleviates menopausal symptoms, such as hot flushes and crow's feet wrinkles (Aso et al., 2012; Oyama et al., 2012).

Currently, the majority of equol production is performed by chemical synthesis, although production of (*S*)-equol via bacterial fermentation may have several advantages over chemical synthesis. Specific intestinal bacteria are responsible for the conversion of daidzein to (*S*)-equol, such as *Coriobacteriaceae* sp. and *Lactobacillus* sp. (Setchell and Clerici, 2010). The Hishigaki group has cloned and identified a gene cluster responsible for converting daidzein to (*S*)-equol from an equol-producing strain *Lactococcus* 20–92 (Shimada et al., 2011, 2012). The gene product of L-*dznr* is responsible for converting daidzein into (*R*)-dihydrodaidzein; the L-*ddrc* gene product converts (*R*)-dihydrodaidzein into (*S*)-dihydrodaidzein; the L-*dhdr* gene product converts (*S*)-dihydrodaidzein into *trans*-tetrahydrodaidze; and the L-*thdr* gene product converts *trans*-tetrahydrodaidzein into (*S*)-equol (Shimada et al., 2011, 2012). Lee et al. (2016) constructed a recombinant *Escherichia coli* BL21 strain which can produce (S)-equol *in vitro*. However, Vázquez et al. (2017) reported that isoflavone-derived compounds like (*S*)-equol have the ability to inhibit the growth from many bacteria species. The aim of this study was to obtain an (*S*)-equol resistant host *E. coli*, which can be engineered for (*S*)-equol production by co-expressing the equol-producing genes L-*ddrc*, L-*dznr*, L-*dhdr,* and L-*thdr*. As a result, a putative oxidoreductase gene *ydiS* was identified to be responsible for the (*S*)-equol resistance. An engineered equol-producing bacterial strain was constructed using an (*S*)-equol resistant mutant [*E. coli* BL21 (*ydiS*)] to coexpress the equol-synthesis genes. A two-step method was utilized to convert diadzin to (*S*)-equol under aerobic conditions. All results of this study have been summarized in a schematic diagram (**Figure 1**).

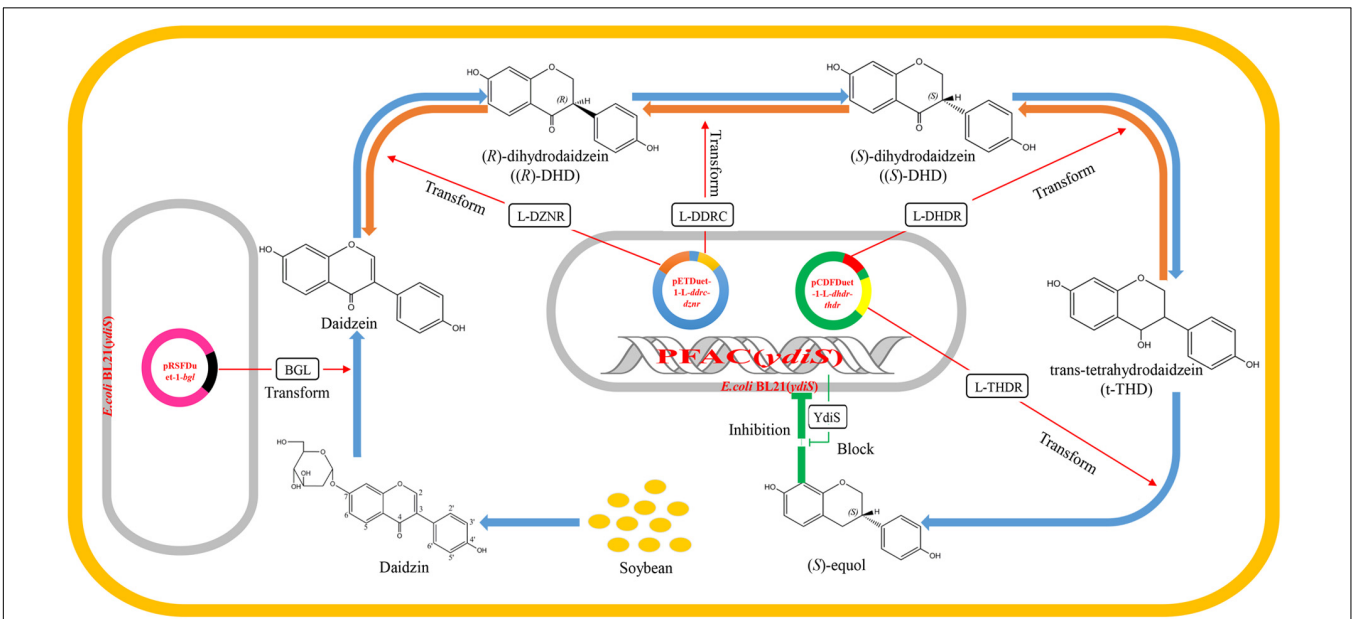

FIGURE 1 | Schematic diagram summarizing the working principle of the engineered equol-producing bacteria. Daidzin is the primary isoflavone that exists in soybean. The first engineered BL21 (*ydiS*) containing *bgl* gene can convert daidzin into daidzein. The fermented liquid was then used as substrate for a second engineered BL21 (*ydiS*) containing equol-producing genes L-*dznr*, L-*ddrc*, L-*dhdr,* and L-*thdr*. The second engineered BL21 (*ydiS*) could then convert daidzein to (*S*)-equol step by step. The engineered BL21 (*ydiS*) could withstand the inhibitory effects of (*S*)-equol by overexpression of a (*S*)-equol resistant gene *ydiS*.

MATERIALS AND METHODS

Chemicals and Reagents

Daidzin, daidzein and (*S*)-equol were purchased from Daicel Chiral Technologies Co., Ltd. (Shanghai, China). The antibiotics and isopropyl-D-thiogalatopyranoside (IPTG) were ordered from Sangon Biotech Bio (Shanghai, China). The restriction enzymes and ligation kit were purchased from TaKaRa Bio (Dalian, China).

Bacteria Strains, Plasmids, and Growth Conditions

Detailed information regarding the strains and plasmids used in this study is listed in Supplementary Table 1. In brief, *E. coli* strains DH5α and BL21 (DE3) were ordered from TaKaRa Bio (Dalian, China) and Transgene Biotech (Beijing, China), respectively. Mariner transposon plasmid pFAC (Wong and Mekalanos, 2000) and a *dap* auxotroph *E. coli* strain WM3064 (Saltikov and Newman, 2003) were obtained from Dr. Gao's laboratory (Zhejiang University, Hangzhou, China). DH5a *E. coli* (pRK2013) was ordered from Biomedal S. L.[1]. The genes L-*dznr* (GenBank accession number: AB558141.1), L-*ddrc* (GenBank accession number: AB694972.1), L-*dhdr* (GenBank accession number: AB592970.1), L-*thdr* (GenBank accession number: AB592969.1), and *bgl* (GenBank accession number: JQ957567.1) were synthesized and then sub-cloned the pUC57 vector by GenScript Biotechnology (Nanjing, China). The *ydiS* gene was PCR amplified using primers *ydiS*-F: 5-ATG TCG GAT GAC AAA TTT GAT GCC A-3, and *ydiS*-R: 5-ATC GCG CCA ACG AGG GAA TTA-3. The *ydiT* gene of BL21 (*ydiS*) was synthesized and sub-cloned into the pRSFDuet-1 vector by GenScript Biotechnology (Nanjing, China). *E. coli* compatible vectors pRSFDuet-1, pETDuet-1, and pCDFDuet-1 were acquired from Merck Millipore (Germany). *E. coli* strains were grown at 37°C in Lennox broth (LB) or LB agar. When required, 50 μg/mL carbenicillin, 50 μg/mL streptomycin, and 15 μg/mL kanamycin were added to the broth or plates. When required, an anaerobic chamber (anaerobic workstation AW 500, Electrotek Ltd., United Kingdom) was employed to minimize oxygen exposure.

Transposon Mutagenesis Library Screening

In this study, the transposon of the pFAC plasmid, consisting of a transposable element flanked by two inverted repeats of 27 bps (5′-aca ggt tgg ctg ata agt ccc cgg tct-3′) and a gentamycin resistance cassette in the middle (aacC1: 534 bp) was used. A gene encoding the hyperactive mariner transposase, and a gene encoding β-lactamase (*bla*) were included in this plasmid (Withers et al., 2014). In theory, the promotor of gentamycin (P_{Gm}) transferred together with the mariner transposon, causing adjacent genes to be overexpressed or repressed, dependent on the transcript directions for the gentamycin promotor and its downstream genes. Transposon mutagenesis was prepared via conjugation utilizing pFAC plasmid-carrying *E. coli* WM3064 as the donor strain and BL21 (DE3) as the recipient strain. Transfer of plasmids from WM3064 to BL21 (DE3) were performed via tripartite conjugations using the helper plasmid pRK2013. In brief, bacterial *E. coli* WM3064, BL21 (DE3), and DH5α (pRK2013) were incubated in LB media at 37°C overnight, 500 μL of each bacterium was then mixed together in a 2 mL tube. After centrifuging, the bacterial pellet was resuspend using LB media and transferred onto a dry LB plate (supplemented with 2,6-diaminopimelic acid) in three compact droplets. After incubation for ∼6 h, the bacteria were gathered and streaked onto LB plates supplemented with gentamicin (15 μg/mL). Bacterial colonies were then seeded into 96-well plates containing LB media supplemented with 200 μg/mL (*S*)-equol. The OD_{600} was detected using a Model 680 microplate reader (Bio-Rad, United States) and SP-2000UV spectrometer (Shanghai Spectrum Instruments Co., Ltd.). The equol-resistant character of the five clones was further verified using a tube culture method. Chromosomal DNA of these (*S*)-equol resistant mutants was isolated using an OMEGA Genomic DNA Extraction Kit (Omega, United States). Taxa identification was performed using by 16s rRNA sequencing and BLAST analysis. The mutant *E. coli* strain BL21 (*ydiS*) was then selected for full genome sequencing using Illumina Hiseq at Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China. The draft genome sequence data of BL21 (*ydiS*) has been deposited in NCBI as Accession Number PIYU00000000.

Detection of Bacterial Growth Rate

For static culture, 30 mL of LB media were added to a 100 mL flask bottle. After autoclaving, bacteria, antibiotics and chemical reagents were then added into the bottle. Bacterial density (OD_{600}) was measured every 3 h. For culturing under shaking conditions, a real-time detection instrument Microscreen-16 (Gering Instrument Manufacturing (Tianjin) Co., Ltd., Tianjin, China) was used. 40 mL of LB media were added to the 50 mL measure bottle, and 400 rpm (equivalent to 100 rpm in the general shake incubator) was utilized for stirring. The optical absorption value was measured at 30-min intervals. Optical absorption was detected at OD_{850}, and a conversion factor between OD_{850} and OD_{600} was calculated using *E. coli* before the experiment.

Batch Culture Fermentation for Equol Production

Batch culture fermentations were cultured without shaking at 37°C, under both anaerobic and aerobic conditions. Briefly, a basic growth medium, LB (Qingdao Hope Bio-Technology Co., Ltd., Qingdao, China), was used to assess the utilization of daidzin or daidzein in the engineered equol-producing *E. coli*. For fermentation, bacteria were aliquoted into 50 mL flask bottles containing 20 mL of culture media supplemented with either daidzin or daidzein. Twenty microliters of IPTG (25 mg/mL) was added to each bottle to induce gene expression. Samples then were collected at 48 and 72 h after IPTG induction to detect equol and daidzein via HPLC. For production of

[1]http://lifescience.biomedal.com/

(*S*)-equol from daidzin, a two-step fermentation was attempted in this study. For the first step, daidzin was transformed to daidzein using *E. coli* (pRSFDuet-1-*bgl*) under fermentation 72 h. The fermentation liquid was then collected after centrifugal separation, and the upper liquid was used for preparing a new LB media (LB-D). For the second step, DDDT-BL21 (*ydis*) was inoculated into the LB-D media to detect the equol production.

HPLC Detection

Identification of equol and daidzein was performed using HPLC according to a previously described method with some modification (Decroos et al., 2005). In brief, 1 mL of each sample was extracted three times with 1 mL acidic ether, then the ether fractions were combined, evaporated to dryness and resuspended in 200 μL of methanol and stored at −20°C until analysis. HPLC analysis was performed using a Waters e2695 system. Fifteen microliter aliquots of each sample were injected and separated using a SunFireTM C18 5 μm column (4.6 mm × 205 mm). The temperature was set at 30 ± 2°C and the flow rate was maintained at 0.8 mL/min. Elution was isocratic with a mobile phase consisting of 0.01% formic acid:methanol:acetonitrile (50:20:30). Equol was detected at 205 nm; daidzein at 254 nm. Calibration curves for the quantification of daidzein and equol were constructed using pure standards obtained from Daicel Chiral Technologies Co., Ltd. (Shanghai, China).

Statistical Analysis

SPSS Software (version 20.0; SPSS Inc., United States) and the Student's *t*-test was employed in this study. $P < 0.05$ was considered to be statistically significant.

RESULTS

Inhibitory Effects of (*S*)-Equol on Host *E. coli* BL21 (DE3)

Previously, (*S*)-equol was shown to inhibit the growth of representative human gut bacteria (Vázquez et al., 2017). However, the inhibitory effects of the fermentation product (*S*)-equol on host bacterial *E. coli* BL21 (DE3) requiring further investigation. In this study, an *E. coli* strain was engineered to coexpress the four equol-producing genes L-*ddrc*, L-*dznr*, L-*dhdr*, and L-*thdr*, which originated from an equol-producing bacterial *Lactococcus* strain 20–92 (Supplementary Figure 1). In order to evaluate the equol-producing activity of the engineered *E. coli*, 50 μg/mL (∼200 μM) daidzein was added to LB culturing media and (*S*)-equol production was detected under both anaerobic and aerobic conditions at different time points after IPTG induction. As demonstrated in Supplementary Figures 2A,D, the metabolites from the engineered *E. coli* had a similar HPLC peaks to the reference standard for (*S*)-equol. The LC-MS results further verified that the equol peak detected by HPLC had the same molecular weight as the (*S*)-equol reference standard (Supplementary Figures 2B,C,E,F). Previous studies have reported that the equol produced by gut bacteria is (*S*)-equol (Setchell et al., 2005), and that the metabolite produced by *Lactococcus* strain 20–92 is (*S*)-equol (Shimada et al., 2011, 2012), the metabolite produced by the engineered *E. coli* was likely to be (*S*)-equol (Supplementary Figure 3). However, bacterial growth rates were inhibited during fermentation (Supplementary Figure 4); bacterial density decreased 30 and 37% under anaerobic and aerobic conditions, respectively, after IPTG was added as an inducer for 24 h. In addition, there was almost no bacterial growth observed even cultured for 72 h (Supplementary Figure 4). In order to clarify which compounds had inhibitory effects on the growth of BL21 (DE3), bacterial plates were prepared using different compounds. The fermentation product (*S*)-equol did inhibit BL21 (DE3) growth both under both anaerobic and aerobic conditions (Supplementary Figure 5), however, daidzein did not cause inhibition. In order to further verify the inhibitory effects of daidzein and equol on bacterial growth, static liquid culture and shake culture experiment were done under aerobic conditions at 37°C. Supplementary Figure 6 illuminated that equol has the ability to inhibit BL21 (DE3) growth which was dependent on the equol concentration. However, daidzein not inhibit growth and may have slightly promoted bacterial growth for both BL21 (DE3) and BL21 (G2) (Supplementary Figures 6A,B).

Screening of (*S*)-Equol Resistant BL21 (*ydiS*) Mutants

The feedback inhibitory effects of the fermentation products could prevent high yields of (*S*)-equol product, transposon mutagenesis libraries were constructed and screened in order to develop (*S*)-equol resistant bacteria. In summary, thounds of bacterial clones grown on LB+Gm plates from the mutant library. 93 clones were then randomly picked up and then seeded in a 96-well plate to evaluate their equol resistance. The growth rate was monitored at different time points using a microplate reader. Among screened clones, five clones were identified as growing faster than BL21 (DE3) in the presence of 200 μg/mL (*S*)-equol (data not shown). Twenty-milliliter tubes containing 5 mL of LB and 5 μL of (*S*)-equol were further used to verify the equol-resistance of these clones (Supplementary Figure 7). 16s rRNA gene sequencing and BLAST analysis were then used for taxonomy analysis of these clones. Mutant *E. coli* strain BL21 (DE3)_G2 [renamed as BL21 (*ydiS*)] was selected for further verification and study. The bacterial density of BL21 (*ydiS*) was higher than BL21 (DE3) under both static and shaken culture conditions in the presence of 50 or 100 μg/mL (*S*)-equol (**Figure 2**). This BL21 (*ydiS*) strain was then selected for equol production and has been deposited into the China General Microbiological Culture Collection Center (CGMCC No. 14219).

Identification of Equol Resistant Gene in BL21 (*ydiS*)

In order to identify the transposon insert site and infer the equol resistant mechanism of BL21 (*ydiS*), full genome scanning and reverse PCR sequencing were performed. The sequencing results revealed that the PFAC transposon was inserted at 307 bp upstream of the *ydiS* gene. The direction

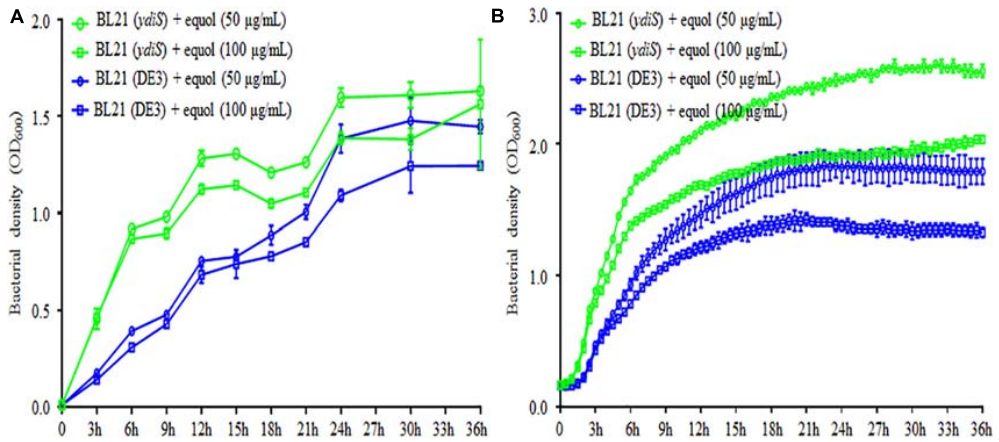

FIGURE 2 | (*S*)-equol resistance of BL21 (DE3) mutant. Growth rates under static **(A)** and shaking **(B)** culture conditions for the BL21 (DE3) and mutant BL21 (*ydiS*) were compared when different concentration of (*S*)-equol were added. At each timepoint, two duplications were measured for each sample, means and standard deviations (SD) were calculated.

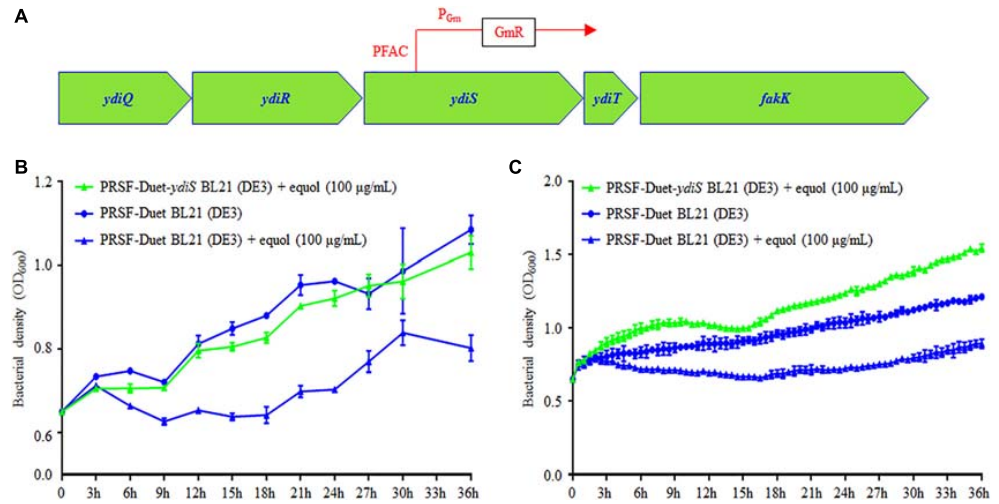

FIGURE 3 | *YdiS* identified as the (*S*)-equol resistant gene in BL21 (*ydiS*). **(A)** Whole genome sequencing revealed that the transposon was inserted at the 307 bp position of the *ydiS* gene; **(B,C)** Overexpression of *ydiS* enabled the *E. coli* BL21 (DE3) to resist (*S*)-equol under static and shaking culture conditions. At each timepoint, two duplications were measured for each sample, means and standard deviations (SD) were calculated.

of PFAC Gm promotor is same as the *ydiS* gene (**Figure 3A**). Overexpression of *ydiS* and its downstream gene *ydiT* may have contributed to the equol resistance for BL21 (*ydiS*). *YdiS* and *ydiT* genes were then cloned into pRSFDuet-1, respectively. When the OD_{600} reached ∼0.6, 5 μL of IPTG (25 mg/mL) was then added to induce foreign protein expression under aerobic condition. As indicated in **Figure 3B**, strains that overexpressed *ydiS* had faster growth rates than pRSF-Duet BL21 (DE3) under both static and shaking culture conditions when supplemented with 100 μg/mL (*S*)-equol. However, overexpressed *ydiT* did not growth faster than the pRSF-Duet BL21 (DE3) when 100 μg/mL (*S*)-equol was supplemented (Supplementary Figure 8). Together, these results indicated that *ydiS* gene was responsible for the equol resistance in the BL21 (*ydiS*) strain.

Comparison of the Equol-Producing Activity of BL21 (DE3) and BL21 (*ydiS*)

No equol production was detected under shaking at 200 rpm (data not shown) and this is consistent with previous reports (Lee et al., 2016). Static culture conditions were utilized to detect the equol production in this study. In order to verify that the mutant strain BL21 (*ydiS*) could grow faster and provide higher yields of (*S*)-equol than BL21 (DE3) during fermentation, the same plasmids pETDuet-1-L-*ddrc-dznr* and pCDFDuet-1-L-*dhdr-thdr* were transformed into BL21 (DE3) [DDDT-BL21 (DE3)] and BL21 (*ydiS*) [DDDT-BL21 (*ydiS*)], respectively. In 50 mL flasks, 20 mL of LB, carbenicillin (final concentration 50 μg/mL), streptomycin (final concentration 50 μg/mL) and two different concentrations of daidzein (5 μg/mL or 50 μg/mL) were added. Before adding IPTG,

the bacterial density was adjusted to OD_{600} = 0.6. After being induced for 48 and 72 h under both anaerobic and aerobic conditions, the bacterial density, daidzein, and equol concentration were measured. The DDDT-BL21 (*ydiS*) was not much better than DDDT-BL21 when 5 μg/mL daidzein was added as the substrate. In contrast, the growth rate, equol yield, and daidzein utilization ratio of DDDT-BL21 (*ydiS*) was greater than DDDT-BL21 (DE3) when 50 μg/mL daidzein was added as substrate both under anaerobic and aerobic conditions (**Figures 4A–C**; Supplementary Figures 9A–C). The daidzein utilization ratio of DDDT-BL21 (*ydiS*) reached 90% under aerobic condition after IPTG induction, while the ratio for DDDT-BL21 was only about 27% (**Figure 4C**). In addition, the higher yield of (*S*)-equol for BL21 (*ydiS*) was not only due to faster growth rates, but also because BL21 (*ydiS*) produced more (*S*)-equol than BL21 (DE3) (**Figure 4D** and Supplementary Figure 9D).

Production of (*S*)-Equol Using Daidzin as the Fermentation Substrate

As daidzin is easily collected from soybean meal, using daidzin as fermenting substrate is extremely convenient. As such, a glycoside hydrolysis gene *bgl 1269* (Gang et al., 2012) was cloned into the compatible vector pRSFDuet-1. The pRSFDuet-1-*bgl* plasmid was then transformed into BL21 (*ydiS*) [PRSF-*bgl*- BL21 (*ydiS*)] to verify the gene's function. After a 72 h induction, daidzin or soybean meal was converted into daidzein under aerobic conditions, with the metabolites from the engineered *E. coli* had a similar HPLC peaks to the reference standard for daidzein (Supplementary Figure 10). Interestingly, after transforming pETDuet-1-L-*ddrc-dznr*, pCDFDuet-1-L-*dhdr-thdr* and pRSFDuet-1-*bgl* into BL21 (*ydiS*), the transformed bacteria could not convert daidzin to (*S*)-equol (data not shown). In order to overcome this hurdle, a two-step method was utilized to transfer daidzin to (*S*)-equol. First, PRSF-*bgl*-BL21(*ydiS*) was used to convert daidzin to daidzein, then the fermentation supernatant was used as substrate for DDDT-BL21 (*ydiS*) to produce (*S*)-equol. After first step fermentation, ∼0.17 mmol/L daidzein was produced from 50 μg/mL daidzin (Supplementary Figure 11A). At this point, if the water prepared for LB media was fully replaced with the fermentation supernatant, (*S*)-equol still was not detected after fermentation (data not shown). However, if 10% of the water was replaced with fermentation supernatant when preparing LB media, ∼0.017 mmol/L (*S*)-equol could be detected after the two-step fermentation under both aerobic and anaerobic conditions (Supplementary Figure 11B).

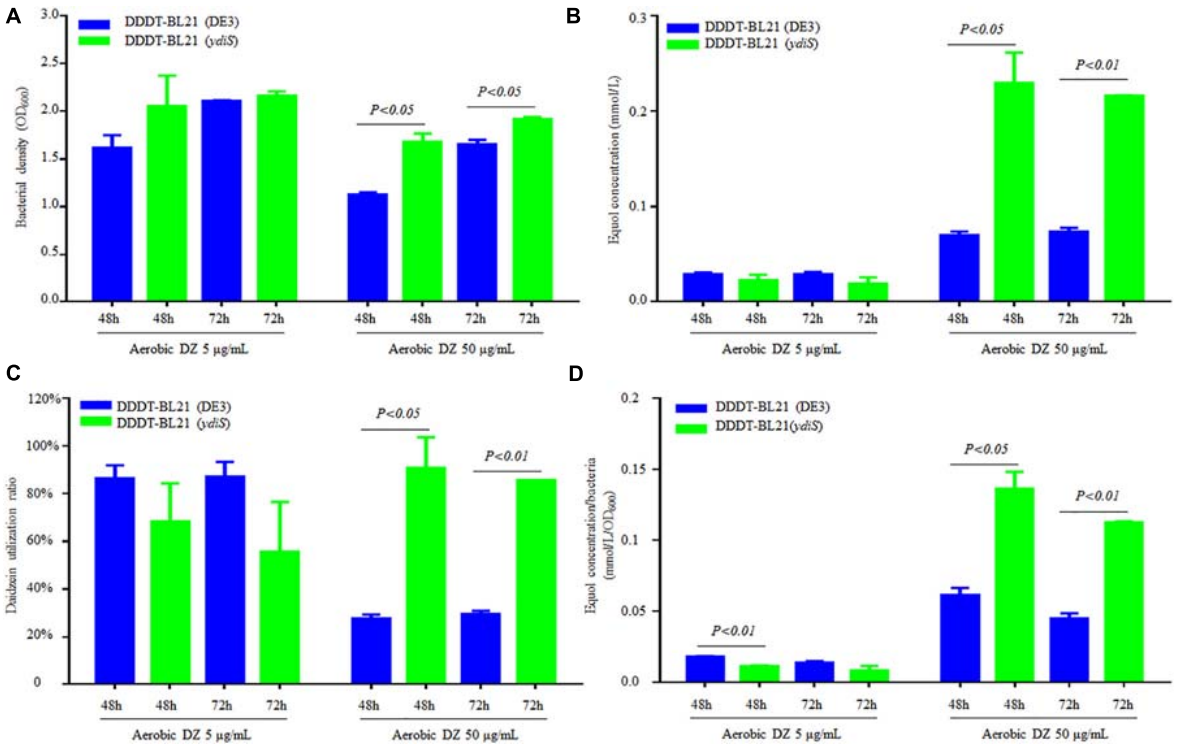

FIGURE 4 | Comparison (*S*)-equol production between DDDT-BL21 (*ydiS*) and DDDT-BL21 (DE3) under aerobic conditions. **(A)** Change in bacterial density; **(B)** Comparison of (*S*)-equol production of DDDT-BL21 (DE3) and DDDT-BL21 (*ydiS*) measured by HPLC; **(C)** Comparison of the daidzein utilization ratio of DDDT-BL21 (DE3) and DDDT-BL21 (*ydiS*); **(D)** Comparison of (*S*)-equol production per bacterium for DDDT-BL21 (DE3) and DDDT-BL21 (*ydiS*). At each timepoint, two duplications were measured for each sample, means and standard deviations (SD) were calculated. The Student's *t*-test was employed in this study, and $P < 0.05$ was considered to be statistically significant.

DISCUSSION

Recently, the gut microbiota has become an intensely researched topic (Garrett, 2017). The microbes in the gut have been recognized as important for proper digestive functions, allowing for a variety of dietary components to be metabolized (Koppel et al., 2017), development of the host immune system (Pamer, 2017), as well as for their impacts on some diseases and infections (Boulange et al., 2016). Dietary components, especially polyphenols have been extensively used as functional food components. Previous research has demonstrated that gut microbiota contribute to polyphenol metabolism and affect its bioavailability (Stevens and Maier, 2016); (*S*)-equol and soy isoflavones are typical examples. (*S*)-equol is the metabolite transferred from soybean meal by gut microbes (Setchell and Clerici, 2010), and the equol hypothesis infers that the main functions of soy isoflavones are due the metabolite product (*S*)-equol (Setchell et al., 2002). Furthermore, the safety of (*S*)-equol has been tested (Liu et al., 2016), indicating that further study of the function and molecular mechanism of (*S*)-equol is important. Heemstra et al. (2006) have developed chemical methods to synthesize (*S*)-equol *in vitro*, but natural (*S*)-equol obtained by microbial fermentation is more attractive, especially as it is produced *in vivo*. Considering the vital function of (*S*)-equol, its production could be important for broad applications. In this study, we constructed an engineered *E. coli* mutant BL21 (*ydiS*) that could convert higher concentration of daidzein to (*S*)-equol under aerobic conditions (**Figure 4**). As daidzin is the main form that is found in soybean meal, (*S*)-equol production from daidzin was attempted by coexpressing the genes *bgl*, L-*ddrc*, L-*dznr*, L-*dhdr,* and L-*thdr*, ultimately producing (*S*)-equol using only a two-step method (Supplementary Figure 11). Coexpression of all five genes in a single system was not sufficient to convert daidzin to (*S*)-equol (data not shown). This phenomenon could have several explanations: (1) glucose generated by *bgl* conversion may inhibit the enzymatic activity. Not only various microbial β-glucosidases reported previously are strongly inhibited by glucose [11–13], intracellular alpha-L-rhamnosidase activity from *Pseudoalteromonas* sp. also affected by the monosaccharides concentration [8]. In addition, during the two-step fermentation equol could be detected 10% but not when it was fully replaced with fermentation supernatant further supporting this hypothesis. (2) host cells were too old to overexpress other genes after converting daidzin to daidzein. (3) the DDRC, DZNR, DHDR and THDR enzymes were deactivitated during the conversion of daidzin to daidzein. Regardless, the fermentation parameters and process need further adjustment.

Polyphenols have inhibitory activity on bacterial growth (Vázquez et al., 2017), which presents challenges when utilizing high yield fermentation to obtain polyphenol products (Chouhan et al., 2017). Although many antibiotic resistance genes have been identified (Liu and Pop, 2009), few studies have investigated polyphenol resistance. In this study, an (*S*)-equol resistant mutant was generated through a transposon mutagenesis screen. Sequencing and overexpression results revealed that *ydiS*, a putative oxidoreductase gene, was responsible for (*S*)-equol resistance (**Figure 3**). Although Bayer et al. reported that complex I NADH oxidoreductase gene (snoD) in *Staphylococcus aureus* affected the susceptibility of thrombin-induced platelet microbicidal protein 1 (Bayer et al., 2006), the equol resistance mechanism of the putative oxidoreductase *ydiS* gene requires further investigation. The inhibitory effects of (*S*)-equol on bacterial growth may be due to its antioxidant function, as the potential oxidoreductase *ydiS* gene product may be counteract redox active of (*S*)-equol, thereby granting equol resistance. Schrettl et al. (2010) have reported similar phenomenon, which they hypothesize that the primary mechanism of gliotoxin inhibits *Aspergillus fumigatus* growth may be via antioxidant activity. Gliotoxin exposure up-regulates several antioxidant-related proteins and elevates superoxide dismutase activity. Moreover, reactive oxygen species production also increases after exposure to gliotoxin. However, glutathione (GSH) levels were significantly elevated in *Aspergillus nidulans* Δ*gliT* compared to wild-type (Carberry et al., 2012). The *gliT* gene encoded a gliotoxin oxidoreductase exhibits a gliotoxin reductase activity, and overexpression of GliT confers protection against exogenous gliotoxin in *A. nidulans* and *Saccharomyces cerevisiae* (Schrettl et al., 2010).

In summary, a putative oxidoreductase gene *ydiS* was identified to be responsible for (*S*)-equol resistance. As a result, an engineered equol-producing bacterial strain was constructed using an (*S*)-equol resistant mutant [*E. coli* BL21 (*ydiS*)] to coexpress the equol-synthesis genes. A two-step method was constructed to convert diadzin to (*S*)-equol under aerobic conditions, providing a new method for (*S*)-equol fermentation and production. In addition, the method used in this study may be useful for screening resistant host cells as an alternative method for production of anti-bacterial components, such as antibiotics and antibacterial peptides. Recently, herbal medicinal remedies have been gaining increased attention, often being combined with probiotics for therapeutic care. However, the inhibitory effects of herbs on probiotics may prevent their application; therefore, screening for polyphenol resistance genes and probiotics engineering could be beneficial for their combined use.

AUTHOR CONTRIBUTIONS

YY and XW conceived and designed the experiments. YY, HL, SM, and HC performed the experiments. YY, HL, LZ, and WL analyzed the data. YY, HL, and XW wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01182/full#supplementary-material

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Genome Sequencing of *Streptomyces atratus* SCSIOZH16 and Activation Production of Nocardamine via Metabolic Engineering

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The *Actinomycetes* are metabolically flexible microorganisms capable of producing a wide range of interesting compounds, including but by no means limited to, siderophores which have high affinity for ferric iron. In this study, we report the complete genome sequence of marine-derived *Streptomyces atratus* ZH16 and the activation of an embedded siderophore gene cluster via the application of metabolic engineering methods. The *S. atratus* ZH16 genome reveals that this strain has the potential to produce 26 categories of natural products (NPs) barring the ilamycins. Our activation studies revealed *S. atratus* SCSIO ZH16 to be a promising source of the production of nocardamine-type (desferrioxamine) compounds which are important in treating acute iron intoxication and performing ecological remediation. We conclude that metabolic engineering provides a highly effective strategy by which to discover drug-like compounds and new NPs in the genomic era.

Keywords: *Streptomyces atratus* ZH16, in-frame deletion, metabolic engineering, siderophore, nocardamine

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INTRODUCTION

Microbially produced natural products (NPs) are one of the most important classes of compounds known to mankind having a vast assortment of applications in medical and agricultural sectors (Bérdy, 2005). It has been estimated that most major classes of antibiotics and over 70% of anti-cancer small molecule therapeutics are microbial NPs, their derivatives, or related congeners/analogs (Newman and Cragg, 2016). With the recently noted rise in resistance to antibiotics and cancer chemotherapeutics, it has become increasingly obvious that novel bioactive NPs are urgently needed to ensure the success of new drug discovery and development initiatives. Genomics and metabolomics have played central roles in ensuring that these needs get met.

Over the last two decades significantly improved technologies for genome sequencing have made it much easier to sequence full microbial genomes. Additionally, studies of microbial genomes have made clear that many microorganisms have far greater potential to produce specialized metabolites than previously thought (Rutledge and Challis, 2015). It has been estimated

that the genomes of species from *Streptomyces* family members, the largest bacterial genus house on average, about 20 biosynthetic gene clusters (BGCs) coding for NPs (Nett et al., 2009). However, over 80% of these gene clusters are typically orphaned under normal laboratory culturing conditions (Nett et al., 2009; Baltz, 2017). Consequently, metabolic engineering and genome mining methods have increasingly been applied to discover secondary metabolites whose corresponding BGCs are normally silent; such BGCs are also sometimes considered "orphan BGCs" to convey the absence of a correlatable NP/s (Zerikly and Challis, 2009; O'Connor, 2015; Baltz, 2016).

Microbial siderophores biosynthesis can generally be classified into two main pathways: non-ribosomal-peptide synthetase (NRPS)-dependent and siderophore synthetase super-family (Barry and Challis, 2009); both pathways are exploited by a range of genera belonging to the *Actinomycetes* (Wang et al., 2014). Many siderophores are NRPS dependent family NPs, such as griseobactin (Patzer and Braun, 2010), coelichelin (Challis and Ravel, 2000), oxachelin (Sontag et al., 2006), as well as tsukubachelin (Kodani et al., 2011, 2013), peucechelin (Kodani et al., 2015), and chlorocatechelins (Kishimoto et al., 2014). Nocardamine is the representative one of the siderophore synthetase super-family (Stoll et al., 1951; Hossain et al., 1983; Ueki et al., 2009). Nocardamines (also called desferrioxamines), composed of alternating dicarboxylic acid and diamine units, originally isolated as antibacterial metabolites from a *Nocardia* strain (Stoll et al., 1951). The BGC responsible for desferrioxamines G_1 and E in *Streptomyces coelicolor* A3(2) was investigated by Barona-Gómez et al. (2004). Among their findings was that the *des* operon contained a subset of four genes coding for the production of various desferrioxamines (Barona-Gómez et al., 2004); their production was found to be regulated by both iron concentrations and by an iron-dependent regulatory protein-IdeR (Günter et al., 1993; Ueki et al., 2009).

Streptomyces atratus SCSIO ZH16 is a deep sea-derived *Streptomyces* that predominantly produces ilamycins under standard laboratory conditions; the biosynthesis of ilamycins has been elucidated in our previous studies (Ma et al., 2017). By applying a combination of Frameplot 3.0 beta (Ishikawa and Hotta, 1999) and AntiSMASH 3.0 (Weber et al., 2013), two online software systems, we were able to predict that up to 26 BGCs are housed within the genome of *S. atratus* SCSIO ZH16; we envisioned that the majority of these are orphan clusters. Accordingly, we applied metabolic engineering methods to activate these putative orphan/silent clusters *en route* to the production of new compounds with potential applications in drug discovery and bioremediation. Here we report: (i) the complete genome sequence of *S. atratus* SCSIO ZH16 as well as a comparative analysis to get further insights into genetic elements involved in biosynthesis of NPs, (ii) the construction of in-frame deletion mutant *S. atratus* SCSIO ZH16S and *S. atratus* SCSIO ZH16NS, and (iii) the identification and structural characterization of nocardamine. Our study highlights the enabling power of metabolic engineering to generate new NPs encoded by orphan gene clusters and also validates the engineered *S. atratus* ZH16NS as a promising nocardamine-based siderophore producer.

EXPERIMENTAL SECTION

General Experimental Section

All bacteria, plasmids and primers used in this work are listed in Supplementary Tables S1, S2. The antibiotics and reagents were purchased from Sangon Biotech Co., Ltd. (Shanghai, China), the PCR polymerase and related reagents were purchased from Takara Biotechnology Co., Ltd. (Dalian, China), gel recycle and PCR recycle kits were purchased from Omega Bio-tek Inc. (Norcross, GA, United States). All solvents were analytical or chromatographic grade and purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China) and Thermo Fisher Scientific Inc. (Waltham, MA, United States).

Column chromatography (CC) was carried out using normal phase silica gel (100–200 mesh, Jiangyou, China) and reverse phase C18 silica gel (40–63 μm, Merck, Germany). Medium-pressure liquid chromatography was performed with a CHEETAH 100 automatic flash chromatography system (Bonna-Agela, China) with an ODS-A flash column (S-50 μm, 12 nm; 100 mm × 20 mm, YMC, Japan). Semi-preparative HPLC was carried out using an Agilent 1260 liquid chromatography system with diode array detector (DAD) (Agilent, United States) and YMC-Pack ODS-A column (250 mm × 20 mm, 5 mm, YMC, Japan). NMR spectra were performed with an Advance 700 MHz spectrometer (Bruker, Germany). High-resolution mass spectral data was obtained from a MaXis quadrupole-time-of-flight mass spectrometer (Bruker, Germany). All the gene amplification was performed with Eppendorf mastercycler pro PCR equipment (Eppendorf, Germany).

Genome Sequencing Bioinformatic Analysis

The collection, identification, and genome sequencing of *S. atratus* SCSIO ZH16 has been previously described (Ma et al., 2017). BGCs and their related ORFs were analyzed by antiSMASH 3.0[1] and Frame Plot 3.0 beta[2], respectively. Furthermore, functional gene annotations and sequence alignments were carried out using Basic Local Alignment Search Tool[3].

In-Frame Deletion of Ilamycin Genes

To obtain NPs encoded by other orphan/silent gene clusters using metabolic engineering methods in *S. atratus* SCSIO ZH16, genetic engineering mutants with clean metabolic background were constructed. IlaS has been identified as a large non-ribosomal peptide synthetase responsible for the incorporation of

[1]https://antismash.secondarymetabolites.org/#!/start

[2]http://www0.nih.go.jp/~jun/cgi-bin/frameplot.pl

[3]https://blast.ncbi.nlm.nih.gov/Blast.cgi

amino acid building blocks to form the full-length heptapeptide. IlaN encoding a cytochrome P450 monooxygenase and IlaO encoding a prenyltransferase were involved in the biosynthesis of L-3-nitrotyrosine and *N*-(1,1-dimethyl-1-allyl)-tryptophan building blocks, respectively. In-frame gene deletions were achieved by following the REDIRECT protocol (Gust et al., 2003). The *S. atratus* SCSIO ZH16 genomic cosmid library was constructed as previously reported (Ma et al., 2017). The apramycin resistance gene *oriT*/*aac(3)*IV fragment was obtained by using specific primers that contain additional *Spe*I restriction sites, and used to replace the target genes in the cosmids 2-10E or 4-07H (Supplementary Table S2). Restriction digests of mutant cosmids with *Spe*I and subsequent relegation predictably abolished the apramycin resistance gene *oriT*/*aac(3)*IV fragment. The second round of PCR-targeting was performed to replace the kanamycin resistance gene on SuperCos I with another apramycin resistance gene *oriT*/*aac(3)*IV fragment obtained by a primer pair ARK (Zhang et al., 2013). The constructed mutant cosmids were introduced into non-methylating *Escherichia coli* ET12567/pUZ8002 and then transferred into *S. atratus* SCSIO ZH16 by conjugation. Because the strain was sensitive to apramycin, exconjugants were grown on solid apramycin containing ISP-4 medium to select for the chromosomal integration of the inactivation constructs. To ensure loss of the target gene from the chromosome, exconjugants were replica-plated once onto antibiotic-free ISP-2 plates. Single colonies were again replica-plated onto apramycin-containing ISP-2 plates and antibiotic-free ISP-2 plates. Apramycin-sensitive clones were evaluated by PCR to ensure proper generation of the desired mutant clones. Two in-frame deletion mutants, *S. atratus* SCSIO ZH16S and *S. atratus* SCSIO ZH16NS, were obtained using this method.

Fermentation and Isolation

The *S. atratus* SCSIO Zh16 wild-type, *S. atratus* SCSIO ZH16S, and *S. atratus* SCSIO Zh16NS mutant strains were cultured in 250 mL flasks containing 50 mL Am2ab liquid media consisting of 0.5% soluble starch, 2% glucose, 0.2% yeast extract, 0.2% peptone, 0.5% soybean meal, 0.05% $MgSO_4.7H_2O$, 0.05% KH_2PO_4, 0.4% NaCl, 0.2% $CaCO_3$, and 3% crude sea salt. Then they were incubated at 28°C on a rotary shaker at 200 rpm for 7 days. Fermentation broths were extracted by 80 mL butanone and the solvent was removed under reduced pressure to give crude extracts that were dissolved in 0.6 mL methanol. Each extract was ultimately subjected to HPLC analysis. The HPLC analysis was carried out with a reversed phase column SB-C18, 5 μM, 4.6 × 150 mm (Aglient, United States) with UV detection at 210 and 285 nm under the following program: solvent system (solvent A, water supplemented with 0.1% trifluoroacetic acid; solvent B, acetonitrile supplemented with 0.1% trifluoroacetic acid); 2–98% solvent B (linear gradient, 0–30 min), 98% solvent B (30–35 min), 98–2% solvent B (35.0–35.1 min), 2% solvent B (35.1–40 min); flow rate was set at 1 ml/min.

The large-scale fermentation of *S. atratus* SCSIO ZH16NS was performed using a two-stage fermentation process. The spores grown on ISP-2 supplemented with 3.0% crude sea salt plates were incubated in a total of 63 flasks (250 mL volume) containing 50 mL Am2ab liquid media at 28°C on a rotary shaker at 200 rpm for 60 h. The 25 mL seed cultures were then transferred into 1 L flasks containing 200 mL Am2ab medium with supplemental 3.0% XAD-16 resin. The resin-containing mixtures were then cultured at 28°C on a rotary shaker at 200 rpm for 8 days. Aliquots (10 mL) were removed from each flask on a daily basis and analyzed by HPLC. After 8 days of growth fermentation broths (28 L) were centrifuged to ensure separation of supernatant, mycelium and resin which were extracted sequentially with twofolds volume butanone (3x), acetone (3x), and EtOH (3x), respectively.

The following HPLC analyses, all extracts (butanone, acetone, and EtOH) were combined, solvent removed *in vacuo* and the remaining extracts subjected to silica gel CC using gradient elution with a $CHCl_3$-MeOH mixture (100:0, 98:2-1, 98:2-2, 96:4-1, 96:4-2, 94:6-1, 94:6-1, 92:8, 9:1,85:15, 8:2,1:1, 0:100) to give 13 fractions (Fr.A1–Fr.A13). Fr.A7 was purified by MPLC with ODS column, which was eluted from 0 to 60% solvent B (A: H_2O, B: CH_3CN) over the course of 60 min to obtain 14 fractions (Fr.J1–Fr.J14). Fr.J2-Fr.J3 were combined and further purified by Sephadex LH-20 chromatography eluted by MeOH and semi-preparative HPLC eluted from 5 to 20% solvent B over 10 min and 20% solvent B over 5 min at a flow rate of 2.5 mL/min using a detection wavelength of 210 nm.

Structural Elucidation

Nocardamine: white acicular crystal, ^1H and ^{13}C NMR data (**Table 1**). HRESIMS: m/z 601.3556 ($[M+H]^+$ calcd 601.3553), m/z 623.3402 ($[M+Na]^+$ calcd 623.3384).

RESULTS AND DISCUSSION

Genome Sequencing and Annotation of *Streptomyces atratus* SCSIO ZH16

Genome sequence information is playing a progressively more important role in NPs discovery as well as studies to elucidate NP biogenesis. Many antibiotic producing strains have been sequenced and examinations of their genomic data have revealed

TABLE 1 | The ^1H NMR and ^{13}C NMR data of nocardamine [In $CDCl_3$ and MeOD (1:1), 700 MHz ^1H NMR and 175 MHz ^{13}C NMR in δ ppm].

Position	δ_C	δ_H (mult, *J* in Hz)
1, 12, 23	–	–
2,13, 24	174.5 CO	–
3, 14, 25	31.3 CH_2	2.51 (2H×3, t, $J = 7.2$)
4, 15, 26	28.5 CH_2	2.80 (2H× 3, t, $J = 7.2$)
5, 16, 27	173.9 CO	–
6, 17, 28	–	–
7, 18, 29	39.7 CH_2	3.20 (2H× 3, t, $J = 6.5$)
8, 19, 30	29.1 CH_2	1.54 (2H× 3, m)
9, 20, 31	23.9 CH_2	1.33 (2H× 3, m)
10, 21, 32	26.5 CH_2	1.65 (2H× 3, m)
11, 22, 33	48.1 CH_2	3.63 (2H× 3, t, $J = 6.5$)

their full biosynthetic potentials which are often far exceeded initial expectations. To better understand the full secondary metabolic potential of *S. atratus* SCSIO ZH16, its genome was sequenced using a combination of 2nd-generation 454 and Illumina HiSeq 4000 sequencing technologies and 3rd-generation PacBio sequencing technology at Shanghai Biozeron Co., Ltd. The assembled genome, along with elucidation of its GC content enabled its classification as a *Streptomyces*. The *S. atratus* SCSIO ZH16 genome is 9,641,288 bp long and consists of a linear chromosome with an average GC content of 69.5% (**Figure 1**). The genome contains 9245 coding sequences, 18 rRNA genes and 69 tRNA genes for transfer of all 20 amino acids (Supplementary Table S3). The genome sequence of *S. atratus* SCSIO ZH16 has been deposited in the Genbank database with the accession number of CP027306.

To elucidate the gene clusters encoded in its genome, the assembled genome sequence was subjected to analysis for secondary metabolite BGCs using online antiSMASH software (see footnote text 1) (Weber et al., 2013) and Frameplot 3.0 beta (Ishikawa and Hotta, 1999). These analyses revealed 26 gene clusters within the *S. atratus* SCSIO ZH16 genome, including six NRPS, four PKS (Type I, Type II, and Type III), four hybrid PKS-NRPS, four terpene, three bacteriocin, two siderophore and three other categories BGCs (Supplementary Table S4) indicating the great potential of the strain to produce an array of secondary metabolites. Significantly, only one gene cluster responsible for ilamycin biosynthesis has been characterized and elucidated from the wild-type strain (Ma et al., 2017). This strain will serve as a target for further genome mining of secondary metabolite BGCs.

Construction of Ila Gene Cluster In-Grame Deletion Mutants and the Discovery of a New Peak in the Genetic Engineered Mutant

Secondary metabolites may be overlooked due to low production levels, a large metabolic background or unpropitious culture conditions (Scherlach and Hertweck, 2009); indeed such considerations have inspired the term "orphan" instead of "silent" BGCs – it is not that a BGC is completely inactive, rather its product simply has not been identified. In our previous report, we have identified IlaS as a large non-ribosomal peptide synthetase responsible for the incorporation of amino

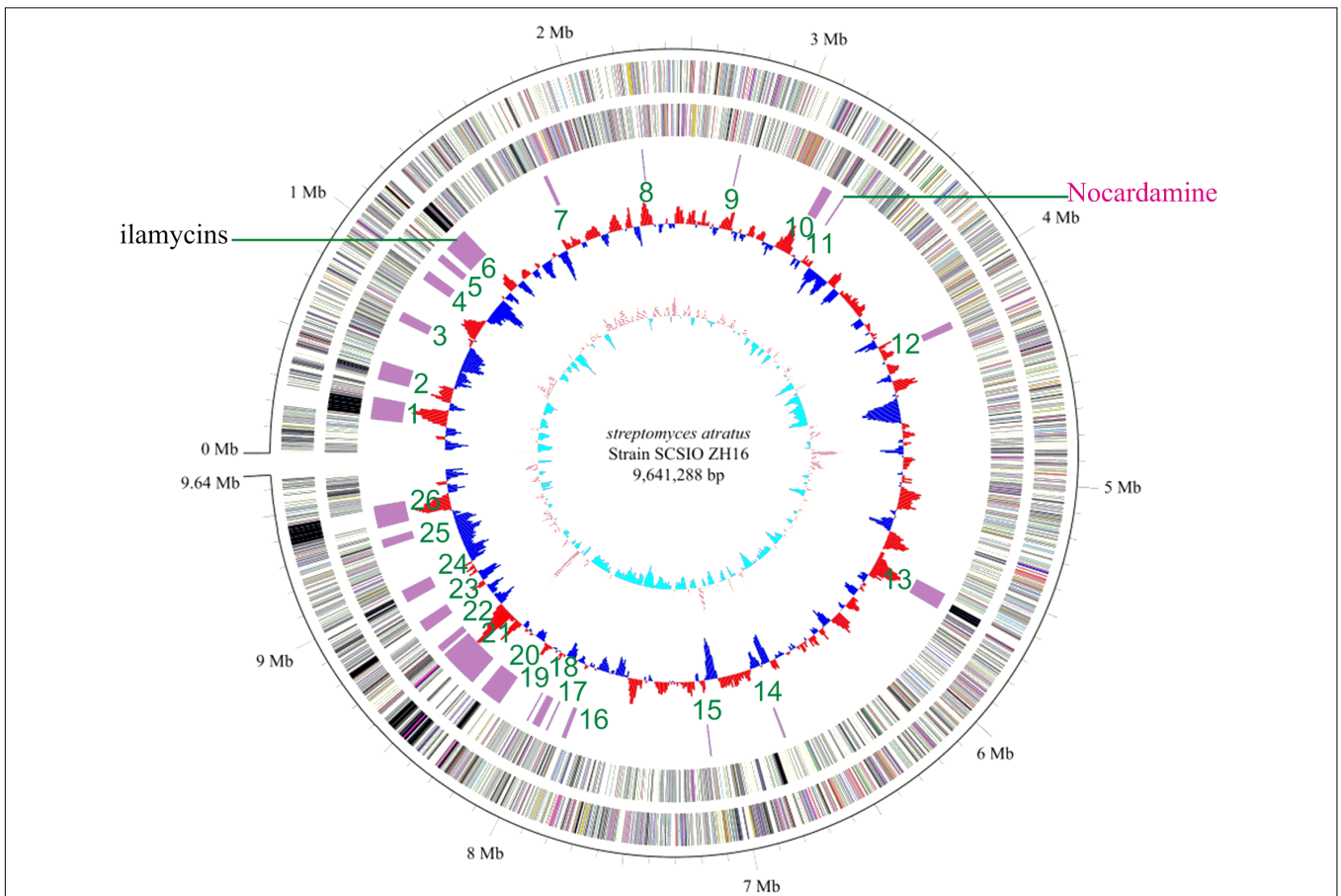

FIGURE 1 | The complete genome of *Streptomyces atratus* SCSIO ZH16. The five circles (outer to inner) represent forward strand CDSs, reverse strand CDSs, nomenclature, and locations of predictive secondary metabolites generated using antiSMASH 3.0 software, GC content and GC skew. Putative nocardamine cluster herein referred to cluster *noc*.

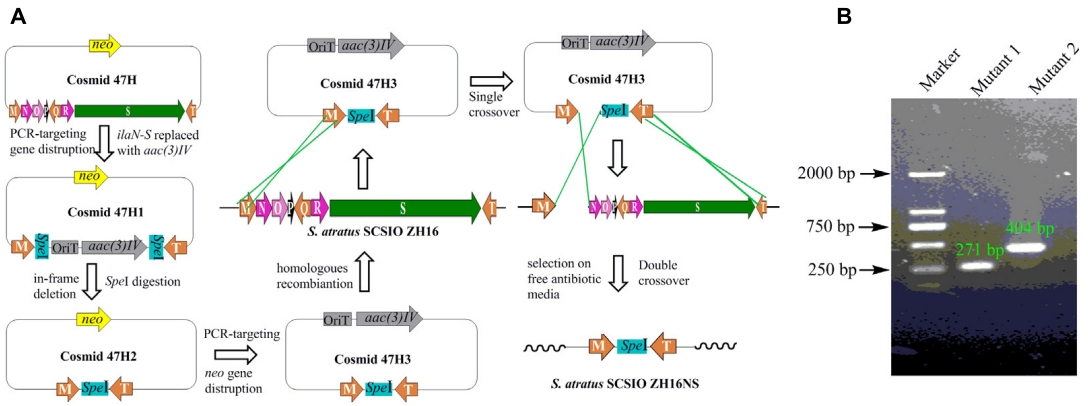

FIGURE 2 | Construction the in-frame gene deletion mutants of *S. atratus* SCSIO ZH16. **(A)** Depiction of constructing in-frame deletion mutant *S. atratus* SCSIO ZH16NS. The 8 kb fragment on cosmid 47H was replaced by the apramycin resistance gene *aac(3) IV* using λ-Red mediated recombination. The resistance marker was cut by *spel* digestion to afford a mutated cosmid47H2 harboring a *spel* sites. Then kanamycin resistance gene was replaced by the apramycin resistance gene, leading to mutated cosmid 47H3. After the conjugation of mutated cosmid 47H3 with *S. atratus* SCSIO ZH16, exconjugant colonies were screened for single crossover mutants by using apramycin resistance. Single recombination colonies were grown on free antibiotic plates to promote double crossover mutant leading to the loss of the target genes. The construction process of in-frame deletion mutant, *S. atratus* SCSIO ZH16S, was the same with that of *S. atratus* SCSIO ZH16NS. **(B)** DNA gel electrophoresis of PCR products obtained with in-frame mutant strain (M: maker, Mutant1: *S. atratus* SCSIO ZH16S, Mutant2: *S. atratus* SCSIO ZH16NS).

acid building blocks to form the full-length heptapeptide of ilamycins (Ma et al., 2017). To enable the production of potential secondary metabolites from *S. atratus* SCSIO ZH16, an in-frame deletion mutant *S. atratus* SCSIO ZH16S (**Figure 2**), devoid of the 1.5 kb *ilaS* gene was constructed to abolish production of the ilamycins, a predominant product of wild-type *S. atratus* SCSIO ZH16 (**Figure 3**, i). Subsequent HPLC analyses revealed that, indeed, ilamycins biosynthesis was abolished upon deletion of *ilaS* (**Figure 3**, ii). However, the ilamycins precursor *N*-(1,1-dimethyl-1-allyl)-tryptophan generated by IlaO from tryptophan was identified in the fermentation extracts of the Δ*ilaS* mutant strain. The generation of a clean background strain for genome mining was not possible. We have demonstrated that IlaN (a cytochrome P450 monooxygenase) is involved in the biosynthesis of L-3-nitrotyrosine building block, and that IlaO (a prenyltransferase) is responsible for the biosynthesis of *N*-(1,1-dimethyl-1-allyl)-tryptophan building block (Ma et al., 2017). Therefore, we sought to further reduce the metabolic background of a genetically engineered *S. atratus* SCSIO ZH16 by carrying out further gene deletions. The in-frame deletion mutant *S. atratus* SCSIO ZH16NS was generated via deletion of an 8-kb fragment spanning from *ilaN* to *ilaS* (**Figure 2**). This mutant strain failed to produce ilamycins as well as the previously noted tryptophan-derived ilamycin precursor (**Figure 3**, iii) and consequently served as an excellent starting strain for the genome mining of *S. atratus* SCSIO ZH16.

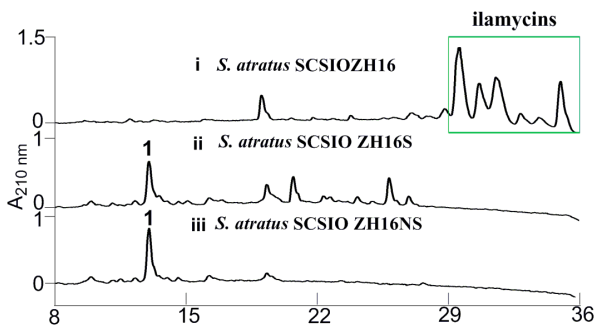

FIGURE 3 | HPLC analyses of metabolite profile for different *S. atratus* strains. (i) *S. atratus* SCSIO ZH16 wild-type; (ii) *S. atratus* SCSIO ZH16S in-frame mutant; (iii) *S. atratus* SCSIO ZH16NS in-frame mutant. 1 is nocardamine.

In order to analyze the metabolomic differences between genetic engineered mutants and their wild type predecessor *S. atratus* SCSIO ZH16, HPLC chromatograms of extracts from the fermentations of the three strains were carefully compared. Notably, a new peak was identified uniquely in the *S. atratus* SCSIO ZH16S and *S. atratus* SCSIO ZH16NS mutants. HRESIMS analysis of the signal generating species revealed a low molecular weight NP with $[M+H]^+ = 601.3556$, $[M+Na]^+$ of 623.3402 (Supplementary Figure S1); on the basis of these data this mutant specific compound was assigned a molecular formula of $C_{27}H_{48}N_6O_9$.

Fermentation, Isolation, and Structural Elucidation of Nocardamine From *S. atratus* SCSIO ZH16NS

To isolate and elucidate the structure of the newly generated NP, large-scale fermentation of *S. atratus* SCSIO ZH16NS was carried out by using 28 L of Am2ab liquid media with a two-step fermentation process as previously reported (Ma et al., 2017). A compound peak retention time of 13.2 min was apparent when using detection at 210 nm; this signal correlated perfectly to the species originally identified

on analytical scale fermentations/analysis with *S. atratus* SCSIO ZH16NS. After several rounds of silica column isolation and medium pressure preparative HPLC, 13.3 mg of analytically pure compound was obtained. For elucidating the structure of the purified compound, the NMR analysis was carried out. The NMR data revealed that ^{1}H and ^{13}C NMR data (**Table 1** and Supplementary Figures S2, S3) which were consistent with the nocardamine spectral data reported in the literature (Yuan et al., 2010). So the compound was identified as nocardamine (also called desferrioxamine E) (**Figure 4**).

Bioinformatic Analysis of the Nocardamine Gene Cluster and the Proposed Biosynthetic Pathway of Nocardamine in *S. atratus* SCSIO ZH16

Further to identify the gene cluster likely responsible for nocardamine biosynthesis, all clusters encoded in the genome of *S. atratus* SCSIO ZH16 were surveyed. Of the 26 unidentified gene clusters, there are two siderophore gene clusters, while cluster 11 showed a high degree of similarity to the previously published desferrioxamine B BGC (Barona-Gómez et al., 2004; Ueki et al., 2009). Accordingly, we assigned this cluster as the nocardamine (desferrioxamine E) BGC. The detailed annotation of the nocardamine gene cluster in *S. atratus* SCSIO ZH16 was postulated and found to contain a *noc* operon with a subset of four genes, enough to code for the biosynthesis of nocardamine (desferrioxamine E). More rigorous BLAST analysis showed that the four genes *nocABCD* in *S. atratus* SCSIO ZH16 encode for pyridoxal decarboxylase (*nocA* gene), putative monooxygenase (*nocB* gene), *N*-acetyltransferase (*nocC* gene), and *IucA*/*IucC* family siderophore biosynthesis protein (*nocD* gene) having 83, 77, 60, 73% identity with *desA*, *desB*, *desC*, *desD* in *S. coelicolor* M145, respectively (**Table 2**). The BGC for desferrioxamine has been reported or characterized in another two *Streptomyces* strains, *Streptomyces avermitilis* K139 (Ueki et al., 2009), and *S. pristinaespiralis* HCCB10218 (Li et al., 2015). Gene functions and organization were identical in each desferrioxamine BGC. The location and organization of the nocardamine gene cluster in the chromosome of *S. atratus* ZH16 were shown in **Figures 5A,B**.

Based on the proposed functions of the genes in *noc* gene cluster, the biosynthetic pathway of nocardamine was proposed as follows: firstly, L-lysine was decarboxylated to yield cadaverine (**2**) by L-2,4-diaminobutyrate decarboxylase encoded by *nocA*. Secondly, cadaverine is hydroxylated at an amino group by monooxygenase encoded by *nocB*, to form *N*-hydroxy-cadaverine (**3**). Then, *N*-hydroxy-cadaverine was condensed with a succinyl-CoA to generate the *N*-hydroxy-*N*-succinylcadaverine (HSC, **4**). Finally, three HSC units were catalyzed to form nocardamine by LucA/Iuc family siderophore biosynthesis protein encoded by *nocD*. The proposed biosynthetic pathway of nocardamine in *S. atratus* SCSIO ZH16 was shown in **Figure 5D**.

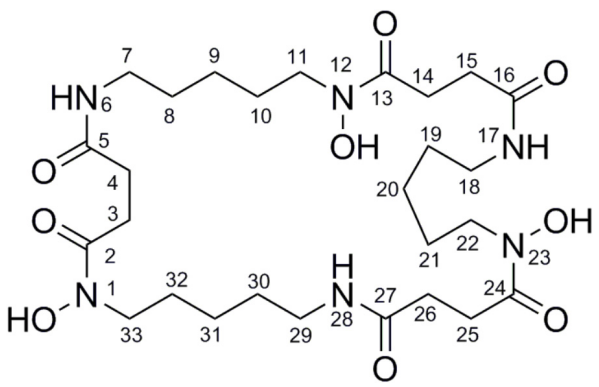

FIGURE 4 | The structure with the numbered C and N atom has been submitted to our journal system to replace this one.

The Possible Regulation of Nocardamine Production in *S. atratus* SCSIO ZH16

The production of nocardamine is carefully controlled by a regulatory protein termed iron-dependent regulatory protein (IdeR) (Günter et al., 1993). To investigate whether the production of nocardamine is also regulated by an IdeR ortholog, three *ideR* genes from *Streptomyces davawensis* strain JCM 4913, *Mycobacterium tuberculosis* H37Rv, and *S. coelicolor* A3(2) were used as probes for BLAST searching within the genome sequence of *S. atratus* SCSIO ZH16. BLAST results indicated

TABLE 2 | Deduced function of individual *orfs* within the *noc* cluster from *Streptomyces atratus* ZH16.

Protein	Size (aa)	Proposed function	Protein homologue, origin[a], ID/SI (%)	Protein homologue, origin[b], ID/SI (%)
ORF(-2)	119	Hypothetical protein	–	–
ORF(-1)	283	Siderophore-interacting protein	–	–
NocA	493	L-2,4-diaminobutyrate decarboxylase	DesA, 83/89	SGR_4750, 88/93
NocB	425	Putative monooxygenase	DesB, 77/86	AlcA, 85/91
NocC	194	*N*-acetyltransferase	DesC, 60/70	AlcB, 76/82
NocD	592	lucA/lucC family sidero-phore biosynthesis protein	DesD, 73/82	AlcC, 84/90
ORF(+1)	553	Hexosaminidase	–	–
ORF(+2)	308	Tat pathway signal sequence domain protein	–	–

ID/SI: Identity/Similarity; [a]Streptomyces coelicolor M145; [b]Streptomyces griseus subsp. griseus NBRC 13350.

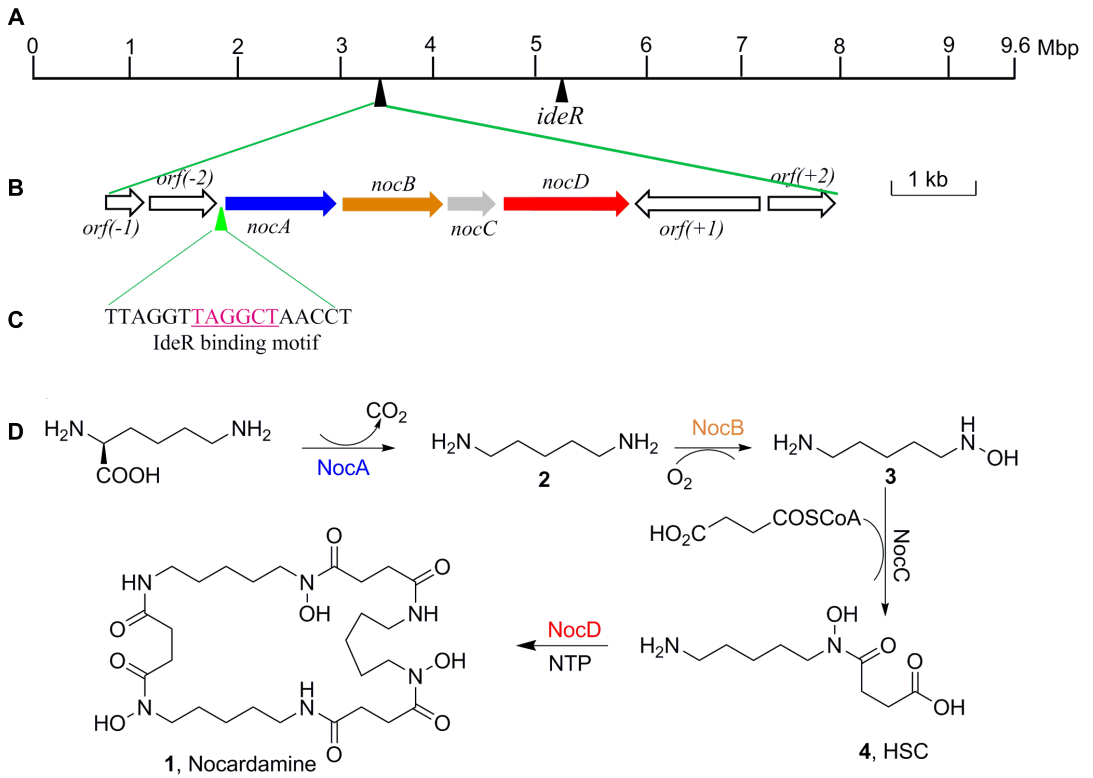

FIGURE 5 | The organization of nocardamine biosynthetic gene cluster and the IdeR binding motif sequence. **(A)** The locations of nocardamine biosynthetic gene cluster and the *ideR* gene. **(B)** The organization of nocardamine biosynthetic gene cluster. **(C)** The magenta sequence was the binding motif of IdeR. **(D)** The proposed biosynthetic pathway of nocardamine.

that a *ideR* ortholog located at 5.1 Mbp of *S. atratus* SCSIO ZH16 chromosome genome has the highest identity (87%) and coverage (88%) with that from the *S. davawensis* strain JCM 4913, and has 86 and 80% identity with that from the *S. coelicolor* A3(2) and *M. tuberculosis* H37Rv, respectively. These results indicated that the production of nocardamine in *S. atratus* SCSIO ZH16 is likely regulated by *ideR* homologues. In order to further determine the binding motif of a putative IdeR regulator, the upstream sequence of the operon *nocABCD* was analyzed by comparison the binding motif to those previously reported in the literature (Günter et al., 1993; Ueki et al., 2009). These comparisons indicated that a 17 base pair sequence "TTAGGTTAGGCTAACCT" has the same IdeR binding motif reported in *S. avermitilis* K139 (Ueki et al., 2009), which is also a nocardamine producer. The regulatory function of IdeR in the production of nocardamine by *S. atratus* SCSIO ZH16 will be characterized in forthcoming publications. The location of the *ideR* gene in the chromosome of *S. atratus* SCSIO ZH16 was shown in **Figure 5A**.

CONCLUSION

The deep sea-derived *S. atratus* SCSIO ZH16 has a linear genome chromosome whose average GC content is 69.5%. Twenty-six gene clusters housed in the genome endow the strain a potential target for genome mining of bioactive NPs. An orphan BGC (*noc*) coding for a siderophore has been activated via metabolic engineering; this entailed the construction and ensuing metabolite analyses of the mutant strains of *S. atratus* SCSIO ZH16S and *S. atratus* SCSIO ZH16NS. These mutants result from efforts to knock out selected portions of the well-studied ilamycin gene cluster. The in-frame deletion mutant of *S. atratus* SCSIO ZH16NS with a relative clean metabolic background, provides excellent opportunity to further mining other orphan gene cluster encoding NPs. The siderophore was structurally elucidated using a combination of HRESIMS and NMR analyses and shown to be previously reported nocardamine. By virtue of its excellent metal ion complexation abilities, nocardamine can be used as an iron carrier to relieve metal toxicity. This work highlights the notion that shifting metabolic flux of an NP producing wild-type strain away from the predominant product pathway may enable the production of new metabolites that, otherwise, are simply not attainable. We posit that nocardamine production (*noc* activation) is enabled at the expense of ilamycin biosynthesis by virtue of engineered shifting of the *S. atratus* metabolic flux. Studies to further dissect this system of engineered cluster activation in the unique marine-derived microbe will be published in due course.

AUTHOR CONTRIBUTIONS

JM and JJ designed the experiments and revised the manuscript. YL analyzed the BGC sequences, performed the mass fermentation of *S. atratus* SCSIO ZH16NS, isolated the compound, and prepared the draft manuscript. CZ constructed two in-frame deletion mutants of *S. atratus* SCSIO ZH16S and *S. atratus* SCSIO ZH16NS and wrote part of the draft manuscript. CL analyzed the NMR data. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

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CRISPR/Cas9-Based Editing of *Streptomyces* for Discovery, Characterization, and Production of Natural Products

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Microbial natural products (NPs) especially of the *Streptomyces* genus have been regarded as an unparalleled resource for pharmaceutical drugs discovery. Moreover, recent progress in sequencing technologies and computational resources further reinforces to identify numerous NP biosynthetic gene clusters (BGCs) from the genomes of *Streptomyces*. However, the majority of these BGCs are silent or poorly expressed in native strains and remain to be activated and investigated, which relies heavily on efficient genome editing approaches. Accordingly, numerous strategies are developed, especially, the most recently developed, namely, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) system reveals remarkable higher accuracy and efficiency for genome editing in various model organisms including the *Streptomyces*. In this mini review, we highlight the application of CRISPR/Cas9-based approaches in *Streptomyces*, focus on the editing of BGCs either *in vivo* or *in vitro,* as well as target cloning of large-sized BGCs and heterologous expression in a genetically manipulatable host, for discovery, characterization, reengineering, and production of potential pharmaceutical drugs.

Keywords: natural product, *Streptomyces*, biosynthetic gene cluster, genome editing, CRISPR/Cas9

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INTRODUCTION

Streptomyces species are known for the most prolific antibiotic producers and have provided a large number of clinical drugs during past decades. However, discovery of natural product (NP) drugs from these talented bacteria has suffered a blow after the Golden Age of NP discovery in 1950s–1960s, that is severely influenced by high-throughput screening of synthetic libraries and the low efficiency of traditional top-down screening strategies (Li and Vederas, 2009). Recently, great advances in next-generation sequencing technologies and computational resources reacquaint microbial genomes and are regarded as a huge reservoir of untapped NP biosynthetic gene clusters (BGCs; Rutledge and Challis, 2015; Weber and Kim, 2016; Kim et al., 2017); moreover, a vast majority of uncultured microorganisms in environments provide limitless possibilities for NP drugs discovery (Banik and Brady, 2010; Katz et al., 2016). For *Streptomyces*, the most gifted bacteria are supposed to possess 20–50 BGCs in a single genome, that greatly exceed the identified compounds (Challis, 2014; Baltz, 2017). Nevertheless, most of BGCs are silent or poorly expressed in native hosts under conventional laboratory culture conditions. To activate these cryptic BGCs, high-efficient approaches for genome editing and BGC engineering garner widespread attention and become a rapidly advancing field for NP drugs discovery (Hsu et al., 2014; Rutledge and Challis, 2015; Choi and Lee, 2016; Jakociunas et al., 2016; Li et al., 2017a; Ren et al., 2017; Zou et al., 2018).

Compared with other model organisms, like *Escherichia coli* and *Saccharomyces cerevisiae*, *Streptomyces* strains show poverty in genetic manipulation and most are recalcitrant for genome editing. In *Streptomyces*, recombinase-mediated homologous recombination has been commonly used for genome editing; however, the related protocols are often laborious and time-consuming (Gust et al., 2003; Komatsu et al., 2010; Fernandez-Martinez and Bibb, 2014; Li et al., 2017a). Until recently, application of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) system, especially the CRISPR/Cas9 system, has greatly facilitated high-efficiency genome editing (Jinek et al., 2012; Choi and Lee, 2016). Likewise, CRISPR/Cas9-based genome editing approaches have greatly accelerated insights into *Streptomyces* derived NP drugs. In this mini review, we summarize the recent developments and challenges of CRISPR/Cas9-based approaches for editing BGCs of *Streptomyces*; moreover, cloning and assembly of intact BGCs for heterologous expression are also emphasized.

CRISPR/Cas9 ADVANCES THE GENOME EDITING

CRISPR/Cas system functions as adaptive immune system in numerous bacteria and archaea, of which RNAs harboring "spacer" sequence from previously exposed bacteriophages help Cas proteins recognize and cleave the specific exogenous DNA (Barrangou et al., 2007; Grissa et al., 2007; Horvath and Barrangou, 2010). Since CRISPR/Cas system exhibits higher specificity and accuracy on sequence targeting, it has become excellent choice for precision genome editing (Jinek et al., 2012). CRISPR/Cas9, a type II CRISPR/Cas system, originally employs CRISPR RNA (crRNA) and *trans*-activating crRNA (tracrRNA) to form crRNA-tracrRNA duplex and then assists Cas9 nuclease to recognize and cleave target DNA harboring trinucleotide protospacer adjacent motif (PAM) and a 5' end of 20 nucleotides complementary to the spacers (Deltcheva et al., 2011; Jinek et al., 2012; Hsu et al., 2014; Nishimasu et al., 2014). System reprogramming that fuses crRNA and tracrRNA into a synthetic single guide RNA (sgRNA) greatly facilitates preparation of transcripts and significantly promotes the application of CRISPR/Cas9 system (Jinek et al., 2012; Hsu et al., 2014). Reprogrammed CRISPR/Cas9 system has since been successfully used in a variety of organisms, including *S. cerevisiae* (DiCarlo et al., 2013), *Drosophila melanogaster* (Gratz et al., 2013), *Caenorhabditis elegans* (Friedland et al., 2013), plants (Jiang et al., 2013), and human embryos (Baltimore et al., 2015).

IN VIVO STRATEGIES FOR GENOME EDITING IN *STREPTOMYCES*

Streptomyces are of utmost importance for novel NP drugs discovery, of which the investigating process relies heavily on high-efficiency genome editing. In *Streptomyces*, classic genome editing commonly achieves through homologous recombination with a suicide or temperature-sensitive or self-replicative plasmid, and requires intensive and time-consuming screening process. The application of CRISPR/Cas9 system for genome editing in *Streptomyces* started in 2015, and since then related approaches have been tremendously developed. As shown in **Table 1**, diversified approaches are widely used to edit or refactor BGCs for NP drugs discovery and characterization.

Cobb et al. (2015) first introduced CRISPR/Cas9 system for genome editing in *Streptomyces*. The pCRISPomyces-2 system equips a codon-modified Cas9 nuclease driven by a strong promoter, a sgRNA expression cassette, and a 2 kb homology repair template (HRT). It first specifically generates a double-strand break (DSB) at target site by Cas9 nuclease under the guidance of sgRNA harboring a custom-designed spacer, and then repairs the resulting chromosome break by homology-dependent repair (HDR) system in the presence of HRT and introduces chromosomal deletions ranging from 20 bp to 31 kb with an efficiency ranging from 70 to 100% (**Figure 1A**). Multiplex genome editing may be achieved by equipping multiplex sgRNA cassettes and corresponding repairing templates in a pCRISPomyces system, and excision of 31 kb BGC of undecylprodigiosin (Red) in *Streptomyces lividans* 66 has thus successfully obtained.

Slightly afterward, three different groups successively applied CRISPR/Cas9 system in *Streptomyces* for diverse applications. The pKCcas9dO system by Lu group similarly revealed high editing efficiency of CRISPR/Cas9 system in *Streptomyces coelicolor* M145 for single gene/BGC deletion, as well as multiplex genes/BGCs deletions (Huang et al., 2015). Besides, a point mutation editing strategy that CRISPR/Cas9 cleaves chromosome DNA at specific site guided by synthetic sgRNA, and then the HDR in *S. coelicolor* helps repair DSB in assistance of HRT with designed point mutation (AAG of 262–264 nucleotides in *rpsL* was changed to GAA), has performed to convert Lys88 to Glu in *rpsL* (**Figure 1A**). Tong et al. (2015) have thoroughly investigated editing efficiency when repairing Cas9-generated site-specific DSBs by non-homologous end joining (NHEJ) system in *S. coelicolor* A3(2). It revealed an incomplete NHEJ system in *S. coelicolor* that lacking a core component LigD, and led to randomly sized deletions around target site. Reconstitution of this defective NHEJ system by complementing *Streptomyces carneus* derived ScaligD has increased editing efficiency up to 77% and qualified the mutations to 1–3 bp deletion/insertion/substitution in most cases (**Figure 1B**). Moreover, high precision genome editing efficiency near 100% achieved when supplying the HRT. In the study, CRISPRi using a catalytically inactive Cas9 nuclease (dCas9) has also been developed, to target promoter region or open reading frame of *actIORF1* for reversible regulation of actinorhodin production in *S. colicolor*. In the same year, Sun group developed an extraordinary CRISPR/Cas9-CodA(sm) combined system, using CodA(sm), the D314A mutant of cytosine deaminase to convert 5-fluorocytosine to toxic 5-fluorouracil, as an efficient counter-selection approach to select for progenies lost recombinant plasmid, which greatly accelerates screening process (Zeng et al., 2015). Besides, a most significant feature that differs from above three systems is application of a segregationally unstable *sti*$^{-}$ pIJ101-derived shuttle vector. The behavior of self-replication

TABLE 1 | Application of CRISPR/Cas9 strategies for genome/BGC editing in *Streptomyces* and some rare actinomycetes.

	Methods	BGCs	Function	Host (original/surrogate)	References
In vivo editing strategy	pCRISPomyces system	Phosphinothricin tripeptide	Deletion	*S. viridochromogenes*	Cobb et al., 2015
		Macrolactam, Lanthipeptide	Deletion	*S. albus*	
		Red, Actinorhodin (ACT)	Deletion	*S. lividans*	Cobb et al., 2015; Wang et al., 2016
		Eumelanin	Deletion	*Actinoplanes* sp. SE50/110	Wolf et al., 2016
		Formicamycins	Deletion	*S. formicae*	Qin et al., 2017
		Oxytetracycline	Site mutation/Deletion	*S. rimosus*	Jia et al., 2017
	pKCcas9dO system	ACT, Red, Ca2 + -dependent antibiotic (CDA)	Deletion/Site mutation	*S. coelicolor*	Huang et al., 2015
		BGC13	Replacement	*S. pristinaespiralis*	Li et al., 2017b
		Cryptic type I polyketide, Red, CDA	Replacement	*S. coelicolor*	
	pCRISPR-Cas9 system	ACT	Deletion	*S. coelicolor*	Tong et al., 2015
		Sceliphrolactam	Deletion	*Streptomyces* sp. SD85	Low et al., 2018
		Dynemicin	Deletion	*Micromonospora chersina*	Cohen and Townsend, 2018
	pCRISPR-dCas9 system	ACT	Reversible regulation	*S. coelicolor*	Tong et al., 2015
	CRISPR/Cas9-CodA(sm) combined system	ACT	Deletion	*S. coelicolor*	Zeng et al., 2015
	CRISPR–Cas9 knock-in strategy	Indigoidine	Promoter insertion	*S. albus*	Zhang et al., 2017
		ACT, Red	Promoter insertion	*S. lividans*	
		Alteramide A, Polycyclic tetramate macrolactam, FR-900098, type I polyketides	Promoter insertion	*S. roseosporus*	
		type III polyketide	Promoter insertion	*S. venezuelae*	
		Pentangular type II polyketide	Promoter insertion	*S. viridochromogenes*	
Direct cloning	CATCH	Jadomycin	Cloning	*S. venezuelae*	Jiang et al., 2015
		Chlortetracycline	Cloning	*S. aureofaciens*	
BGC refactoring in yeast	mCRISTAR	Tetarimycin, Lazarimide, AB1210	Promoter refactoring	*S. albus*	Kang et al., 2016
In vitro editing strategy	ICE	RK-682	Deletion/Insertion	*S. lividans*	Liu et al., 2015
		Holomycin	Deletion	*S. albus*	
		Tü 3010	Deletion	*S. avermitilis*	Tao et al., 2016
	CRISPR/Cas9 system combined with Gibson assembly	Pristinamycin II	Vector refactoring	*S. pristinaespiralis,*	Li et al., 2017b
		Chloramphenicol	Vector refactoring	*S. coelicolor*	

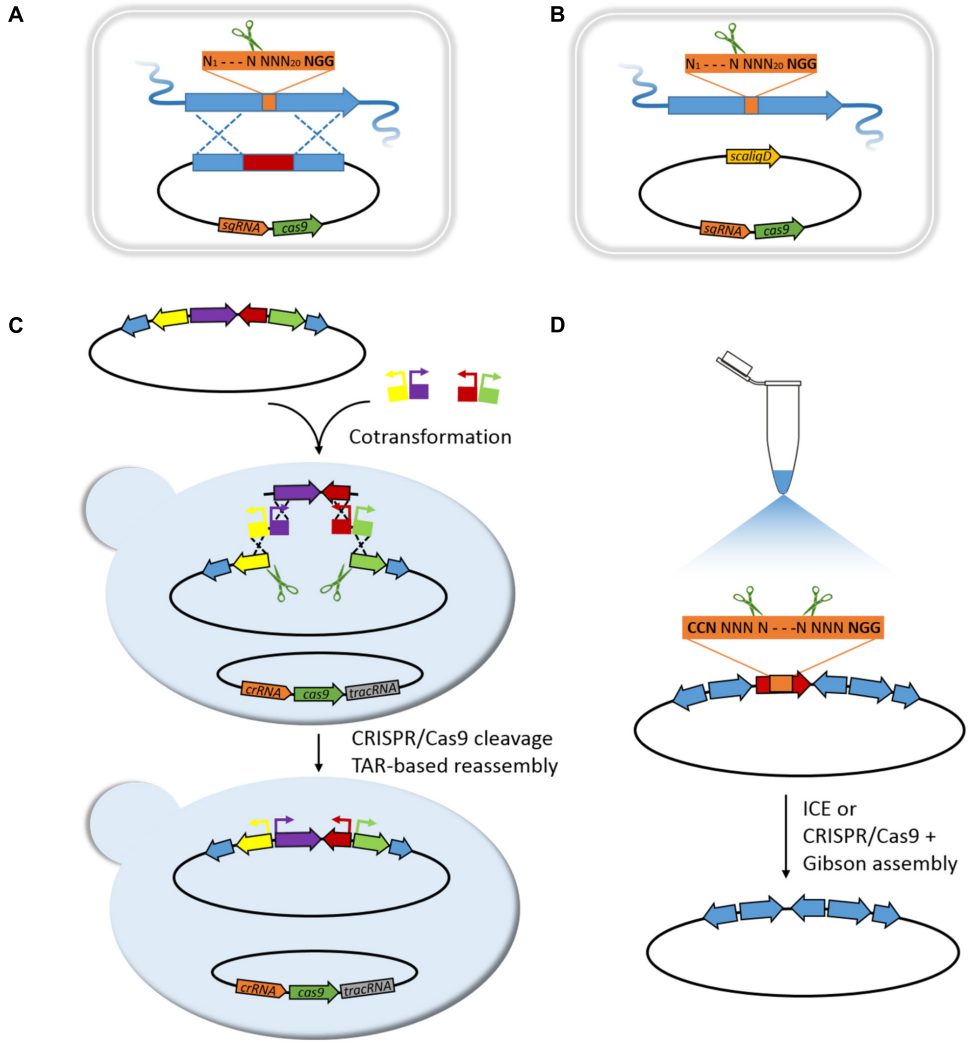

FIGURE 1 | Editing of BGCs based on CRISPR/Cas9 strategies. **(A)** HDR-mediated editing in *Streptomyces*. Gene deletion, point mutation, or promoter substitution can be performed, respectively, when the repairing template carries corresponding deletion, point mutation, or promoter replacement. Multiplex loci editing can be achieved by equipping multiplex sgRNA cassettes and corresponding HRTs. **(B)** NHEJ-mediated editing in *Streptomyces*. Small sized deletion, insertion, or substitution (mostly 1–3 bp) close to the target site can be achieved by using a reconstituted NHEJ system that co-expressing the *scaligD* in *S. coelicolor*. **(C)** mCRISTAR for BGC refactoring in yeast. **(D)** *In vitro* editing of BGC by ICE or SRISPR/Cas9 coupling Gibson assembly.

with high copy number of about 50 per chromosome of this deliver vector can produce large amounts of single-strand plasmid DNA and plenty of template DNAs, resulting in dramatically high efficiency of double cross-over recombination and frequency of target mutant (Zeng et al., 2015). All the above reports facilitate rapid progress for genome editing in *Streptomyces*, since CRISPR/Cas9 helps select against wild-type sequence in the presence of HRTs. CRISPR/Cas9 system also enables activation of cryptic BGCs in *Streptomyces*. Zhao group utilized CRISPR/Cas9-mediated knock-in strategy for efficient and precise insertion of constitutive promoters upstream of main biosynthetic operons or pathway-specific activators, and triggered production of novel NPs of different classes in multiple *Streptomyces* species (**Figure 1A**; Zhang et al., 2017).

To date, CRISPR/Cas9 system has been applied for genome editing in *Streptomyces* for 3 years, the high specificity and efficiency made it the most attractive technology in that field. Its application has now extended to many non-model *Streptomyces* strains, like *Streptomyces formicae* from the African fungus-growing plant-ant *Tetraponera penzigi* (Qin et al., 2017), *Streptomyces rimosus* with distinctive chromosome terminal and core regions (Jia et al., 2017), *Streptomyces* sp. SD85 from tropical mangrove sediments (Low et al., 2018), and some rare actinomycetes like *Actinoplanes* sp.SE50/110 (Wolf et al., 2016) and *Micromonospora chersina* (Cohen and Townsend, 2018). However, *in vivo* application of this fascinating technology in *Streptomyces* is confined to the strains are genetically tractable, missing out on a vast amount of precious BGC resources from genetically intractable strains or yet uncultured strains. In that

case, acquiring and refactoring of intact BGCs for heterologous expression in a genetically tractable surrogate host could be alternatively considered.

CRISPR/Cas9-MEDIATED BGCS CLONING AND REFACTORING FOR HETEROLOGOUS EXPRESSION

Cloning of Large-Sized BGCs

A variety of cryptic BGC awakening approaches, like pathway-specific/global regulator manipulation, promoter refactoring, and ribosome engineering, have been used for NPs discovery in *Streptomyces*. However, most require genetic manipulation of native strains thus are constrained in genetically intractable strains or BGCs from environmental DNA (eDNA; Rutledge and Challis, 2015; Weber et al., 2015; Zhang et al., 2016). Strategies for heterologous expression of BGCs in a genetically manipulatable host can perfectly circumvent above bottleneck, but cloning and editing of large-sized BGCs (sometimes over 100 kb) remain challenging. For cloning large-sized DNAs, classic strategies generally utilize randomly digested genomic libraries; however, the screening process is always laborious and it is arduous for packaging intact BGCs over 100 kb in a single vector. Previous precision cloning strategies often utilize restriction enzymes (REs) to release target BGCs that subsequently acquired by coupling diverse DNA capturing strategies. Linear–linear homologous recombination (LLHR) uses RecE/T mediated homologous recombination for direct capture of REs generated genome segments and is widely used for direct cloning of NP BGCs from *Streptomyces* (Fu et al., 2012; Nah et al., 2017). Gibson assembly coupling REs cleavage is also used for capturing BGCs, and accordingly, the conglobatin cluster has target cloned by Leadlay group (Zhou et al., 2015). Transformation-associated recombination (TAR) cloning uses homologous recombination in *S. cerevisiae* to capture REs generated BGC segments, and has employed for cloning BGCs of taromycin A (Yamanaka et al., 2014), alterochromide (Ross et al., 2015), and thiotetronates (Tang et al., 2015). However, these REs-dependent approaches are severely constrained for broader application since appropriate RE cutting sites do not regularly exist close to BGC terminals. CRISPR/Cas9 system perfectly addresses the limitation, that target cleaves the DNA guided by a synthetic sgRNA, allowing target cloning of large-sized BGCs. Wang et al. (2015) tentatively applied CRISPR/Cas9 system as REs *in vitro* to linearize a large vector (22 kb) and subsequently seamlessly assembled with a small DNA using Gibson assembly (Wang et al., 2015). For precision acquiring large-sized DNAs harboring NP BGCs, Jiang et al. (2015) developed Cas9-assisted targeting of chromosome segments (CATCH), which allows target cloning of intact BGCs up to 100 kb that cleaved by CRISPR/Cas9 at specific sites guided by custom-designed sgRNAs and subsequent target captured by Gibson assembly (Jiang et al., 2015). Simultaneously, Lee et al. (2015) combined CRISPR/Cas9 with TAR cloning that employs homologous recombination in yeast to target capture CRISPR/Cas9 released chromosomal segments and dramatically accelerated capture efficiency of TAR cloning up to 32% (Lee et al., 2015). Soon after, CRISPR/Cas9 system coupling TAR cloning was further applied to construct even megabase-sized DNA segments. Zhou et al. (2016) developed Cas9-facilitated homologous recombination assembly (CasHRA), which co-introduces large circular DNAs into *S. cerevisiae* and release the target DNA segments by CRISPR/Cas9 for subsequent assembly by homologous recombination. It provides an alternative for assembly of large-sized BGCs over 100 kb, using DNAs obtained from cosmid libraries of *Streptomyces* or eDNA. However, it involves assembly steps and tends to be time-consuming.

CRISPR/Cas9-Mediated Editing of BGCs

Acquiring of intact BGCs of interest is the first step to heterologously investigate the novel NP drugs. Editing of acquired BGCs is generally required for successful heterologous expression. Routine strategies for BGCs editing are always constrained by difficulty of handling large-sized DNAs, and are always laborious. λ-Red recombination mediated PCR-targeting has often used for editing BGC by creating gene replacements/deletions; however, residues like antibiotic selection markers or FRT sequence remain at editing sites, and unintended recombination may raise from repetitive sequences of such modular PKS or NRPS genes (Gust et al., 2003; Yamanaka et al., 2014). λ-Red recombination also enables promoter refactoring or domains/modules exchange for characterization of NPs biosynthesis (Nguyen et al., 2006; Du et al., 2013). Recently, a more facile promoter refactoring approach based on homologous recombination in *S. cerevisiae* has been developed by Brady group, that enables multiplex promoter refactoring in a single TAR reaction (Montiel et al., 2015). Based on this, production of eDNA-derived indolotryptoline antiproliferative agents, lazarimides A and B, was activated. Nevertheless, the refactoring rate is relatively low. Homologous recombination in yeast could be greatly improved if specific DSBs are introduced at recombination sites (Storici et al., 2003; Storici and Resnick, 2006; Lee et al., 2015). Accordingly, Brady group developed multiplexed-CRISPR-TAR (mCRISTAR) approach, which introduces CRISPR/Cas9 system to specifically create DSBs across target recombination sites (Kang et al., 2016). With mCRISTAR, multiplex CRISPR/Cas9 generated operon fragments can be reassembled with synthetic promoter cassettes by homologous recombination, and are capable of achieving four promoters exchange simultaneously in a single round using one auxotrophic marker selection, with efficiency up to 80% (**Figure 1C**). General applicability of mCRISTAR has been validated by applying to activate three different cryptic BGCs coding for tetarimycin, lazarimide, and AB1210, indicating a powerful and promising technology for discovery of novel NP drugs from cryptic BGCs resource.

In contrast to the above *in vivo* strategies for BGC editing based on homologous recombination in *E. coli* or *S. cerevisiae*, Sun group developed a new *in vitro* CRISPR/Cas9-mediated editing (ICE) system for high-efficient BGCs editing (Liu et al., 2015). ICE system allows a complete *in vitro* operating process with normal molecular operations, which cleaves BGCs at specific sites guided by synthetic sgRNAs and ligates the blunt ends

that are repaired by T4 polymerase, to create gene in-frame deletion/replacement/insertion mutations (**Figure 1D**). With ICE system, BGCs of tetronate RK-682 and dithiolopyrrolone holomycin were readily edited (Liu et al., 2015), especially for Tü 3010, a particular thiotetronate antibiotic, various gene in-frame deletions were rapidly constructed and accordingly deciphered biosynthesis of this exceptional thiotetronate structure (Tao et al., 2016). Soon afterward Lu group utilized a similar *in vitro* approach that coupling CRISPR/Cas9 system with Gibson assembly to refactor the bacterial artificial chromosome vector harboring BGC of pristinamycin II for following multiplexed site-specific genome engineering in *Streptomyces* (**Figure 1D**; Li et al., 2017b). The above two examples indicate that *in vitro* application of CRISPR/Cas9 could be of wide applicability for BGCs editing, especially coupling the subsequent heterologous expression of BGCs for NP drugs discovery, characterization, and engineering. Nevertheless, optimization of *in vitro* strategies for multiplex loci refactoring of BGCs is of great necessity, and coupling of CRISPR/Cas9 system with Gibson assembly may preliminarily address the problem.

CONCLUSION

In conclusion, CRISPR/Cas9 system has proved to be a powerful technology for genome editing or BGC refactoring due to the outstanding features, like higher sequence specificity, artificial guided targeting, and high editing efficiency. Its applications of genome editing specialized for *Streptomyces* are still relatively narrower in range, especially for the strains little studied. Thus, more efficient and convenient CRISP/Cas tools are of urgent requirement. For instance, diversified CRISPR/Cas systems like Cpf1 (Zetsche et al., 2015; Fonfara et al., 2016; Li et al., 2016), the newly identified class 2 type V CRISPR/Cas protein, xCRISPR/Cas9 (Hu et al., 2018), the most recently evolved CRISPR/Cas9 system with broad PAM compatibility, and even the CRISPR/Cas systems from *Streptomyces* (Choi and Lee, 2016) could be introduced for diverse applications in *Streptomyces*, to advance the researches on NP drugs and open a new era for NP drugs discovery.

AUTHOR CONTRIBUTIONS

WT and YS wrote the manuscript. All authors revised and approved the manuscript.

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Metabolic Engineering of *Escherichia coli* for Enhanced Production of Naringenin 7-Sulfate and Its Biological Activities

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Flavonoids are one of the predominant groups of plant polyphenols, and these compounds have significant effects on human health and nutrition. Sulfated flavonoids have more favorable attributes compared to their parent compounds such as increased solubility, stability, and bioavailability. In this research, we developed a microbial system to produce sulfated naringenin using *Escherichia coli* expressing a sulfotransferase (ST) from *Arabidopsis thaliana* (At2g03770). This wild-type strain was used as a model system for testing clustered regularly interspaced short palindromic repeats (CRISPR) interference (CRISPRi) metabolic engineering strategies. Using synthetic sgRNA to mediate transcriptional repression of *cysH*, a gene encoding 3′-phosphoadenosine-5′-phosphosulfate (PAPS) ST, which is involved in sulfur metabolism, resulted in an increase in intracellular PAPS accumulation by over 3.28-fold without impairing cell growth. Moreover, naringenin 7-sulfate production by engineering *E. coli* with its *cysH* gene repressed in the open reading frame through CRISPRi was enhanced by 2.83-fold in compared with the wild-type control. To improve the efficiency of biotransformation, the concentration of SO_4^{2-}, glucose, and substrate were optimized. The bioproductivity of naringenin 7-sulfate was 135.49 μM [∼143.1 mg (47.7 mg L^{-1})] in a 3-L fermenter at 36 h. These results demonstrated that the CRISPRi system was successfully applied for the first time in *E. coli* to develop an efficient microbial strain for production of a sulfated flavonoid. In addition, antibacterial and anticancer activities of naringenin 7-sulfate were investigated and found to be higher than the parent compound.

Keywords: sulfotransferase, CRISPRi, *E. coli*, PAPS, metabolic engineering

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INTRODUCTION

Flavonoids are major natural phenolic compounds found in plants (Buer et al., 2010). One of the most predominant citrus flavonoids is naringenin and it was found to exhibit various biological effects on human health and nutrition. Naringenin showed anti-estrogenic, antioxidant (Bugianesi et al., 2002), anti-obesity and anti-diabetic activities (Hossain et al., 2016). It has been demonstrated to control non-alcoholic steatohepatitis and associated inflammation (Jadeja and Devkar, 2014). Like flavonoids, most of the naringenin in nature accumulates in a glycosylated form (Gattuso et al., 2007), which improves solubility, storage, and stability of the parent compounds

(De Bruyn et al., 2015). In addition to glycosylation, many the sulfate conjugate of flavonoids discovered in the different type of plants (Barron et al., 1998). However, the biological and physiological properties of sulfated flavonoids have not been studied (Totta et al., 2005; Wang et al., 2014).

Sulfation plays important roles in detoxification of xenobiotics (Yi et al., 2006; Chen et al., 2015) and regulating the activity of animal hormones (Geyer et al., 2017). Enzymatic sulfation is catalyzed by a family of sulfotransferases (STs) that transfer the sulfonate group of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to a hydroxyl group or amino group of acceptor compounds with the parallel formation of 3′-phosphoadenosine-5′-phosphate (PAP) (Paul et al., 2012). Although the sequences of many STs have been deposited to various genome databases of plant (Gidda and Varin, 2006; Marsolais et al., 2007), bacteria (Hossain et al., 2011), and mammalian (Wong et al., 2010; Shimohira et al., 2017), the enzymatic and microbial synthesis of sulfonated natural products still are limited. One of the major problems of *in vitro* sulfation reactions is the use of the costly and unstable sulfate donor, PAPS, which impedes industrial scale-up of the process. Moreover, while chemical synthesis of sulfated compounds is tedious and time-consuming in terms of involving multiple steps (Zhang et al., 2012), PAPS has a poor availability in the cytosol of microbial cells like *Escherichia coli* and *Streptomyces* for production or modification of natural compounds (Sekowska et al., 2000; Nishikawa et al., 2017). Recently, genetic engineering has been used for production or post-modification of the natural compound *via* improving the availability of the common precursor in the biosynthetic pathway. For example, metabolic engineering of *E. coli* in the superpathway of methionine and *S*-adenosyl-L-methionine (SAM) biosynthesis, lead to improved SAM availability, and finally increased anthocyanin *O*-methylation (Cress et al., 2017). The sugar pathway was overexpressed to improve the cytoplasmic pool of NDP-sugars and subsequently expressed glycosyltransferase was used for the biosynthesis of glycosylated flavonoids in *E. coli* (Kim et al., 2015; Pandey et al., 2016). However, the same approach has not been applied to the biosynthesis of sulfated flavonoids.

Genetic editing using the clustered regularly interspaced short palindromic repeat (CRISPR) system has been widely used in diverse organisms including bacteria (Huang et al., 2015; Wu et al., 2015), fungal kingdoms (Wang et al., 2017), plant (Khan et al., 2017), animals (Gandhi et al., 2017), and human cell lines (Bertomeu et al., 2017). In the type II CRISPR, an RNA-guide DNA endonuclease (Cas9) is targeted to specific DNA sequences through a chimeric guide RNA (gRNA), which is a fusion between a precursor CRISPR RNAs (crRNA) and trans-activating CRISPR RNAs (tracrRNAs) (Huang et al., 2015). This gRNA is capable of recognizing sequences target sites for marker-free integration or gene disruption that are followed by the protospacer-adjacent motif (PAM) sequence NGG, N being any nucleotide (Cress et al., 2015). Recently, CRISPR interference (CRISPRi) has been developed which contains a mutation in the Cas9 protein (D10A and H840A) resulted in a lack of endonuclease activity (dCas9), but DNA binding capability remained for inhibition of transcription (Bikard et al., 2013). CRISPRi has been applied to down-regulation of genes in certain pathways *via* metabolic engineering of *E. coli* for production of value natural compounds (Wu et al., 2015; Liang et al., 2016; Gao et al., 2017). However, it has not been reported to regulate a gene in *E. coli* sulfur metabolism.

In this study, we expressed a ST from *Arabidopsis thaliana* (At2g03770) for biosynthesis of sulfated naringenin in *E. coli* BL21 (DE3). Naringenin has been shown to be substrate specificity of At2g03770 (Hashiguchi et al., 2013). Furthermore, we employed a CRISPRi system as a tool for improving PAPS availability by knockdown PAPS ST (*cysH*) in the sulfur metabolism (**Figure 1**), resulted in enhancement the final products. This is the first research that a metabolic engineering approach for conjugating a sulfate moiety generated in the cytoplasm of *E. coli*. The biosynthesized compound was also tested for its potential bioactivities against various pathogens and cancer cell lines.

MATERIALS AND METHODS

Chemicals and Reagents

Restriction enzymes, shrimp alkaline phosphatase, T_4 polynucleotide kinase, and T_4 DNA ligase were obtained from New England Biolabs (Hertfordshire, United Kingdom). Standard naringenin, adenosine 3′-phosphate 5′-phosphosulfate lithium salt hydrate (PAPS), adenosine 5′-triphosphate disodium salt hydrate (ATP), adenosine 5′-diphosphate (ADP), deuterium oxide (D_2O), and dimethyl sulfoxide-d_6 (DMSO-d_6) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Isopropyl-β-D-thiogalactopyranoside (IPTG) was obtained from GeneChem Inc. (Daejeon, South Korea). All other chemicals were of the highest grade commercially available.

Plasmid and Strains Constructions

All the *E. coli* strains and plasmids used in this study are supported in **Table 1**. PCR primers utilized for gene amplification and cloning were synthesized by GenoTech Corp. (Daejeon, South Korea). At2g03770 were codon-optimized for *E. coli*, synthesized, cloned into the plasmid vector pUC57 by General Biosystems, Inc. (NC, United States). The *Bam*HI and *Xho*I sites were added to the 5′ and 3′ ends of the gene. The synthesized gene was restricted by *Bam*HI and *Xho*I digestion, subcloned into the expression vector pET28a (+) to create the pET28a(+)-At2g03770. Then, the recombinant plasmid was transformed into *E. coli* BL21 (DE3) to form the strain used for production (wild-type) (**Table 1**).

Plasmids used for CRISPRi/dCas9-mediated transcriptional repression was obtained from Addgene (Plasmid #65006) (Supplementary Figure S1) and constructed as previously reported (Cress et al., 2015). The specific targeting spacer of *cysH* from the *E. coli* K12 genomic DNA was identified near promoter (*cysH1*) and open reading frame region (*cysH2*) to prevent RNAP binding and elongation, respectively. The primer pairs crRNA1-Fw/Rv and crRNA2-Fw/Rv were used to construct CRISPRi-1 and CRISPRi-2 listed in Supplementary Table S1. Both primers were synthesized, phosphorylated with T_4 polynucleotide kinase, and annealed (Cress et al., 2017).

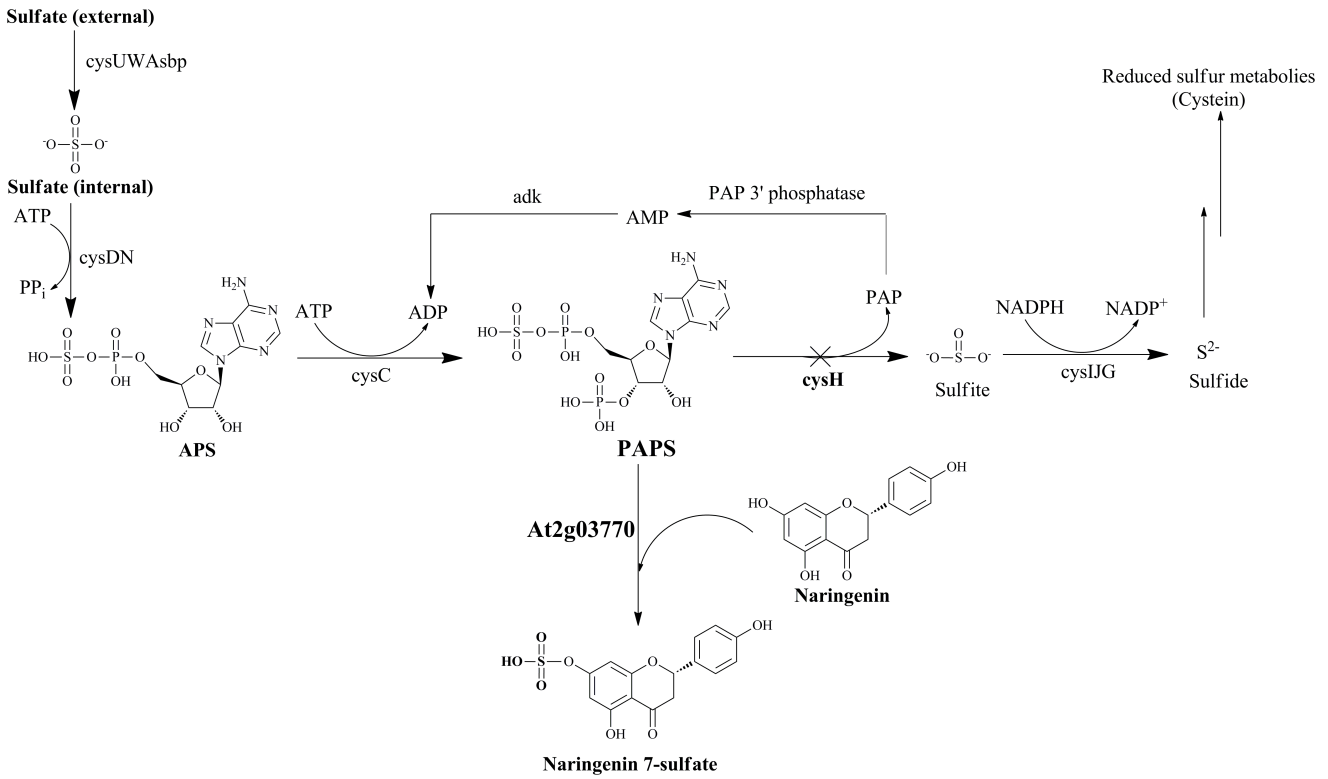

FIGURE 1 | Schematic of sulfur metabolic pathway with strategies for biosynthesis of naringenin 7-sulfate in *E. coli*. *Cross* show the repressed gene in this pathway by using CRISPRi system. Operon *cysUWAsbp*, a sulfate permease; operon *cysDN*, a ATP sulfurylase; *cysC*, APS kinase; *cysH*, PAPS sulfotransferase; operon *cysIJG*, sulfite reductase; *adk*, adenylate kinase; *APS*, adenosine 5′-phosphosulfate; *PAP*, 3′-phosphoadenosine-5′-phosphate.

TABLE 1 | Strains and plasmids used in this study.

Strain/plasmids	Properties/genotype	Source/reference
Strains		
E. coli DH5α	F-Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK−, mK+) phoA supE44 λ− thi-1 gyrA96 relA1	Novagen
E. coli BL21(DE3)	ompT hsdT hsdS (rB^- mB^-) gal (DE3)	Novagen
Wild-type	*E. coli* BL21 (DE3) carrying pET 28a(+)-At2g03770	This study
S1	*E. coli* BL21 (DE3) carrying pET 28a(+)-At2g03770 and pCRISPathBrick-1	This study
S2	*E. coli* BL21 (DE3) carrying pET 28a(+)-At2g03770 and pCRISPathBrick-2	This study
Plasmid vectors		
pET 28a(+)	Expression vector with T7 promoter, p15A ori, Km^r	Novagen
pET 28a(+)-At3g45070	pET 28a(+) carrying At2g03770 from *A. thaliana*	This study
pCRISPathBrick	pACYC184 (Cm^r), p15A ori, *Streptococcus pyogenes* dCas9 (D10A, H840A), tracrRNA, non-targeting CRISPR spacer with *Bsa*I site	Cress et al., 2015
CRISPRi-1	pCRISPathBrick, CRISPR spacer targeting *cysH* near promoter	This study
CRISPRi-2	pCRISPathBrick, CRISPR spacer targeting *cysH* on open reading frame	This study

The products insert were then ligated into *Bsa*I-digested, dephosphorylated, gel-purified CRISPRi plasmid backbone. *E. coli* DH5α was used for cloning experiments. All CRISPRi plasmid arrays possessing synthetic the specific targeting spacer were verified by colony PCR with primer pairs cPCR-Fw/Rv (Supplementary Table S2) and sequencing. Plasmids were constructed with CRISPRi-1 and CRISPRi-2 were transformed into the wild-type strain through calcium chloride and a heat-shock method (Dagert and Ehrlich, 1979), forming variant S1 and S2 strains (**Table 1**).

Culture Conditions and Recombinant Protein Expression

Wild-type *E. coli* was cultured in Luria Bertani (LB) liquid medium. The sample was kept in a 250 mL flask on a 37°C with

shaking incubator 200 rpm. Kanamycin (50 μg mL^{-1}) was used for plasmid selection and maintenance. A total of 250 μL of the pre-inoculum of wild-type *E. coli* was transferred to 50 mL fresh LB liquid medium containing an appropriate amount of antibiotic and then incubated at 37°C with 200 rpm shaking. Protein expression was induced by the different concentration of IPTG (0.1, 0.5, and 1.0 mM) when the optical cell density at 600 nm ($OD_{600\ nm}$) reached 0.6–0.8. The cell growth was continued at 20°C for 20 h and harvested by centrifugation at 842 × *g* for 15 min. The sample was washed twice with 50 mM Tris–HCl (pH 7.5) buffer containing 10% glycerol and prepared for sonication in 1 mL of the same buffer. Following centrifugation at 13,475 × *g* for 30 min at 4°C, the resulting soluble and insoluble protein fractions were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Analysis of Cell Growth and mRNA-Expression Levels

The seed culture of the wild-type and S1, S2 strains were transferred to 50 mL fresh LB medium in a 250 mL flask and $OD_{600\ nm}$ of each flask was diluted to 0.1. All the culture containing chloramphenicol (34 μg mL^{-1}) or kanamycin (50 μg mL^{-1}) were growth under sharking at 37°C with 200 rpm shaking. After 6 h, 0.1 mM IPTG was added to induce the CRISPRi system and the growth was continued at 20°C with 200 rpm shaking for 48 h.

Total RNA was isolated by using RNeasy Plus Mini Kit (Qiagen, United States). The QuantiTect Reverse Transcription Kit (Qiagen, United States) was used to synthesize the cDNA from 1 μg total RNA sample. Power SYBR Green Master Mix (Thermo Fisher Scientific, United States) was performed by quantitative real-time PCR (qRT-PCR) on StepOnePlus real-time PCR system (Applied Biosystems, United States). The 16S rRNA gene was used to an endogenous control with primer pairs 16S rRNA-Fw/16S rRNA-Rv (Supplementary Table S2). The comparative 2^{-Ct} experiment was used to calculate relative gene expression (Livak and Schmittgen, 2001) by using wild-type as the reference sample with primer pairs *cysH*-Fw/*cysH*-Rv. The results were analyzed using StepOne Software v2.3 (Applied Biosystems, United States).

Intracellular PAPS Collection and Quantification

Three recombinant strains including wild-type, S1 and S2 were grown, induced with 0.1 mM IPTG and expressed in 50 mL LB medium at 20°C with 200 rpm shaking for 12 h. Then, the cells were harvested by centrifugation, suspended, and incubated in 250 mL flasks containing 50 mL of M9 minimal salt medium pH 7.4 at 28°C for 12 h. After that, the samples were taken, chilled immediately on ice and centrifuged at 842 × *g* at 4°C for 15 min. One milliliter of 6% perchloric acid was used for cell lysis and 0.3 mL of 3 M potassium carbonate was added to neutralize while vortexing the sample (Fowler et al., 2009). After centrifugation removed the cell residue, the supernatant containing was collected, filtered through a 0.22-μm syringe filter and injected into UFLC-PDA to determine the products yield. The determination of microbial biomass was carried out by dry cell weight using a 0.22-μm syringe filter. An aliquot of 1 mL cell culture was filtered, washed with distilled water, and dried in a conventional oven. DCW was represented by the weight difference between empty membranes and those with cell residues (Wu et al., 2015).

Sulfated Naringenin Production and Extraction

The wild-type and S1, S2 strains harboring heterologous a ST and CRISPRi were first expressed in 50 mL fresh LB medium by 0.1 mM IPTG at 20°C for 12 h. Next, the cells were harvested by centrifugation, washed twice with 100 mM phosphate buffer pH 7.4, and incubated at 28°C with shaking in 250 mL flasks containing 50 mL M9 minimal salt medium pH 7.4 (Yan et al., 2005). Appropriate antibiotics and IPTG were added at the same time. Subsequently, 100 μM substrate was supplemented to the same samples and kept on 48 h. We carried out fermentation in 3-L of optimal media under an optimal condition for large-scale production of naringenin derivatives. The processing of fermentation was followed as previously described (Chu et al., 2016). Furthermore, the culture samples were extracted with twice volume of ethyl acetate. The extracts were concentrated by freezing rotary evaporator, suspended in methanol, and then injected into UFLC-PDA to determine the products yield.

HPLC Analysis

The UFLC-PDA system containing a reversed-phase column [Mightysil RP-18 GP 250-4.6 (5 μm) Cica-Reagent; Kanto Chemical Co., Inc., Japan] was used to separate the samples. The mobile phase containing solvent A (HPLC-grade water consisting 0.05% TFA) and solvent B (100% HPLC-grade methanol) was maintained at 30°C. A calibration PAPS and naringenin standards were created with various concentration.

For quantification of PAPS, the intracellular PAPS was analyzed at a UV absorbance of 254 nm (Uesugi et al., 1997; Xu et al., 2012). The mobile phase including solvents A and B was maintained at 1 mL min^{-1} for 10 min. The program was followed by 10% B for 1 min, 20% B for 1 min, 30% B for 30 s, 35% B for 30 s, 40% B for 1 min, 50% B for 4 min, 90% B for 1 min, and 10% B for 1 min.

Quantification of production was based on the peak areas obtained at 290 nm. The conversion percentage of the substrate was determined after integrating substrate and product peaks. Solvents A and B were run at 1 mL min^{-1} for a 30 min. The gradient of the mobile phase was carried out and followed by 20% B for 5 min, 50% B for 5 min, 70% B for 5 min, 90% B for 5 min, and 10% B for 10 min.

The purification of naringenin derivatives was performed by preparative HPLC (Shimadzu, Tokyo, Japan) with C_{18} column [YMC-Pack ODS-AQ (150 mm × 20 mm I.D., 10 μm)] connected to a UV detector at 290 nm using a 40 min binary program with 5% B for 5 min, 40% B for 5 min, 40% B for

5 min, 90% B for 5 min, and 10% B for 10 min at a flow rate of 10 mL min^{-1}.

Mass Spectrometry and Nuclear Magnetic Resonance

High-resolution quadruple time-of-flight electrospray ionization-mass spectrometry (HR–QTOF ESI/MS) analysis was carried out with an ACQUITY column coupled an SYNAPT G2-S (UPLC, Waters Corp., Billerica, MA, United States). A reversed-phase column [Acquity BEH C_{18} 2.1 mm × 100 mm (1.7 μm)] was used to separate the samples. ESI/MS detection of the samples: positive ion mode ESI^+, acquisition range: 50–1,400 *m/z*, capillary voltage: 2.5 kV, cone voltage: 30 V, source temperature: 120°C, desolvation gas temp: 600°C, cone gas flow: 20 L h^{-1}, desolvation gas flow: 800 L h^{-1}. The analyses were performed at a flow rate of 0.35 mL min^{-1} using the same mobile phase with a gradient of 30% B for initial, 90% B for 4 min, 100% B for 3 min, 100% B for 2.5 min, and 37.5% B for 2.5 min. MassLynx software version 4.1 (Waters Corp.) was used for analysis the chromatograms.

The purified products were dried, lyophilized, and recorded on Bruker Biospin 300 MHz spectrometer in DMSO-d_6 for proton ^{1}H-NMR.

Antibacterial Activity

Nine Gram-positive bacteria and six Gram-negative bacteria listed in **Table 2** were used for testing the antibacterial of naringenin and its derivative. All strains were cultured in LB medium at 37 ± 1°C. We applied the paper disc diffusion method using ampicillin (current antibiotic standard) as an antibacterial positive control for screening the antibacterial agent (Rios et al., 1998). Each inoculates consisted of 10^6 colony forming units (CFU mL^{-1}) and was spread on MHA plates for bio-assay. Sterile filter paper discs (6 mm in diameter) consisting of 10 μL of 100 mM of compounds dissolved in MeOH were spotted on the agar surface. The plates were incubated at 37 ± 1°C and checked for 36 h.

Anticancer Activities

Three cancer cell lines containing A375SM melanoma, MCF-7 breast cancer, AGS gastric cancer, and 267B1 cellosaurus were observed from the Korean Cell Line Bank (KCLB, Seoul, South Korea). Minimum essential medium (MEM) added 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, United States) was used for culture the cell lines at 37°C in a humidified 5% CO_2 incubator. Cells seeded at 2×10^3 cell $well^{-1}$ in 96-well plates (SPL Life Sciences, Gyeonggi, South Korea) were treated with each compound in serial dilution (400, 200, 100, and 50 μM) for 72 h. Taxol, a current anticancer agent, was also carried out to provide an additional point of comparison. We examined cell growth using an MTT colorimetric assay (Lee et al., 2017).

Statistical Analysis

Values are mean ± standard deviation (SD). All the results were the average of three independent experiments with SD (n = 3). Differences with p-values <0.05 and <0.005 were indicated a considered significant.

RESULTS

Escherichia coli Expression of Recombinant *A. thaliana* SULTs

To optimize conditions for the expression of recombinant ST At2g03770 from *A. thaliana*, we induced with various IPTG

TABLE 2 | Inhibition zone diameter (mm) of naringenin and naringenin 7-sulfate against nine Gram-positive bacteria and six Gram-negative bacteria.

No.	Pathogens	Naringenin	Naringenin 7-sulfate	Ampicillin
Gram-positive bacteria				
1	*S. aureus* CCARM 0205 (MSSA)	–	7.0 ± 0.08	7.2 ± 0.09
2	*S. aureus* CCARM 0204 (MSSA)	–	+	+
3	*S. aureus* CCARM 3634 (MRSA)	–	+	23 ± 0.27
4	*Proteus hauseri* NBRC 3851	–	+	20 ± 0.24
5	*Micrococcus luteus* KACC 13377	–	9.5 ± 0.18	15 ± 0.36
6	*Bacillus subtilis* ATCC 6633	–	–	–
7	*Bacillus subtilis* KACC 17047	–	–	–
8	*Enterococcus faecalis* 19433	–	–	13 ± 0.18
9	*Enterococcus faecalis 19434*	–	–	14 ± 0.20
Gram-negative bacteria				
10	*Kocuria rhizophila* NBRC 12708	–	–	16 ± 0.14
11	*Klebsiella pneumoniae* ATCC 10031	–	–	16 ± 0.12
12	*E. coli* ATCC 25922	–	–	30 ± 0.32
13	*Salmonella enterica* ATCC 14028	+	+	13 ± 0.14
14	*Pseudomonas aeruginosa* KACC 10232	–	7.2 ± 0.12	–
15	*Enterobacter cloacae* subsp. *dissolvens* KACC 13002	–	+	9 ± 0.10

–, no inhibition zone; +, inhibition zone detected.

concentrations (0.1, 0.5, and 1.0 mM) under 20°C in LB liquid medium. The SDS-PAGE analysis of the soluble and insoluble fraction of At2g03770 indicated the highest expression level as determined at 0.1 mM IPTG. The recombinant protein, At2g03770 (37.72 kDa), was overexpressed in *E. coli* BL21 (DE3) and obtained in the soluble fraction (Supplementary Figure S2).

Construction of CRISPRi System in *E. coli*

Gene *cysH* was selected for studying CRISPRi system on the sulfur metabolism of *E. coli.* The knockdown of *cysH* might to the increase of the intracellular PAPS pool enhancing production of sulfated substrates. Therefore, two 66 bp complementary offset oligonucleotides containing 30 bp protospacer (target) sequence were designed (Supplementary Table S1) and inserted to pCRISPathBrick, resulted in CRISPRi-1 and CRISPRi-2 systems formation (**Table 1**). The length of the *cysH* gene is 735 bp, while the length of the target sequence is 30 bp. To transcriptional interference, *cysH1* was designed 56 bp upstream from start codon (from position −76 to −57), after the PAM sequence AGG (from position −58 to −56). At the same time, *cysH2* was designed 10 bp downstream of the start codon (from position 40 to 11), after PAM sequence AGG (from position 41 to 43) (**Figure 2A**). The unique properties of *E. coli* BL21 genomic of each two protospacer were confirmed *via* nucleotide BLAST[1]. The plasmids were assembled and then biotransformation into *E. coli* DH5α. Colony PCR (cPCR) was performed with cPCR-Rv and cPCR-Fw primers (Supplementary Table S2). The cPCR products were confirmed through 2% agarose gel with 50 bp DNA ladder marker (ELPIS-Biotech. Inc., South Korea). The length of amplifying obtained from clones of pCRISPathBrick is 85 bp, while a 66 bp increase in PCR obtained from clones of CRISPRi-1 and CRISPRi-2 (Supplementary Figure S3). The plasmids CRISPRi-1 and CRISPRi-2 were transformed into wild-type strain, forming production strains S1 and S2, respectively (**Table 1**).

Effect of CRISPRi System on Cell Density and Gene Expression

We determined and compared the growth curves of wild-type, S1, and S2 strains by using a spectrophotometer (Thermo Fisher Scientific, United States) *via* the $OD_{600\ nm}$. The data showed that the S1 and S2 had to resemble with the wild-type on the rate of cell growth (**Figure 2B**), indicating that *cysH* gene inhibition by CRISPRi did not affect the cell growth. In addition, the relative qRT-PCR data of knockdown S1 and S2 strains were analyzed through *cysH* mRNA quantification using 16S rRNA as an endogenous control and wild-type as the reference sample. 16S rRNA, known as a housekeeping gene, is one of the most commonly used endogenous control in *E. coli* (Zhou et al., 2011). The data showed qRT-PCR cycle threshold values for 16S rRNA gene expressed at almost similar levels in all three samples containing wild-type, S1, and S2 with 23.852 ± 0.371, 22.558 ± 0.031, and 21.732 ± 0.115, respectively. This results demonstrated that the suitability of 16S rRNA gene for the specific case of this experiment (Supplementary Figure S4). Importantly, the value of relative expression of *cysH* gene transcription in wild-type was 1, while this figure for S1 and S2 were 0.312 and 0.081, respectively (**Figure 2C**). The result indicated that both the prevent RNA polymerase binding (CRISPRi-1) and elongation (CRISPRi-2) have high efficiency of the reducing transcriptional expression level of *cysH*.

The Knockdown of *cysH* Gene Increased PAPS and Naringenin Derivatives

The intracellular PAPS in three strains were extracted using the method described above. All the samples consisting authentic ADP, ATP, and PAPS standard were determined by using UHPLC-PDA coupled HR-QTOF ESI/MS. UHPLC-PDA analysis showed the ADP standard appeared at retention time $t_R \sim 0.83$ min, while t_R of ATP and PAPS standard were 2.71 and 1.27 min. Interestingly, the intracellular PAPS was detected at $t_R \sim 1.27$ min in the cytosol of wild-type as well as mutant strains (Supplementary Figure S5). These peaks were confirmed by HR-QTOF ESI/MS and the results were shown in Supplementary Table S3 and Supplementary Figures S6, S7. These results suggested that UHPLC-PDA coupled HR-QTOF ESI/MS not only the distinguishing between PAPS from potential interferents ADP and ATP but also the detection of the intracellular PAPS in the cytosol of *E. coli*. Finally, the intracellular concentration of PAPS was analyzed and shown in **Figure 2D**. While the figure for S1 and S2 strains went up to 40.58 and 74.85 $\mu M\ g^{-1}$ DCW, the concentration of PAPS for wild-type obtained a 22.85 $\mu M\ g^{-1}$ DCW at the same time. This result demonstrated that *cysH* is the most required target to inhibit the PAPS consumption.

Moreover, we used the three recombinant strains to produce the sulfated derivative from naringenin. The UFLC-PDA chromatograms of extract from the whole cell of all strains showed a new peak at retention time $t_R \sim 18.248$ min (P1) in comparison with naringenin standard at $t_R \sim 19.979$ min under UV absorbance at 290 nm (Supplementary Figure S8). These peaks were further analyzed by HR-QTOF ESI/MS. The found mass of naringenin was ∼273.0780 $[M + H]^+$ m/z^+ equivalent to molecular formula $C_{15}H_{13}O_5$ with $\lambda_{max} \sim 287$ nm, for which calculated mass was ∼273.0763 (Supplementary Figure S9A). The found mass of P1 at ∼353.0330 with λ_{max}: 277; 335 nm, corresponding to the exact mass of the mono-sulfate derivative of naringenin with molecular formula $C_{15}H_{12}O_8S$ for $[M + H]^+$ $m/z^+ \sim 353.0331$ (Supplementary Figure S9B). The structural identifies of the product could be verified *via* NMR in the future experiment. The percentages of bioconversion of naringenin to mono-sulfated naringenin were 17.2% in the wild-type strain, while the figure for both S1 and S2 strains showed an enhancement to 32.47 and 48.67% at the same time (**Figure 2E**). These results demonstrated that S1 and S2, both mutant strains include the knockdown of the *cysH* gene were used for the improvement of naringenin derivative. It might be true that S2

[1]https://blast.ncbi.nlm.nih.gov

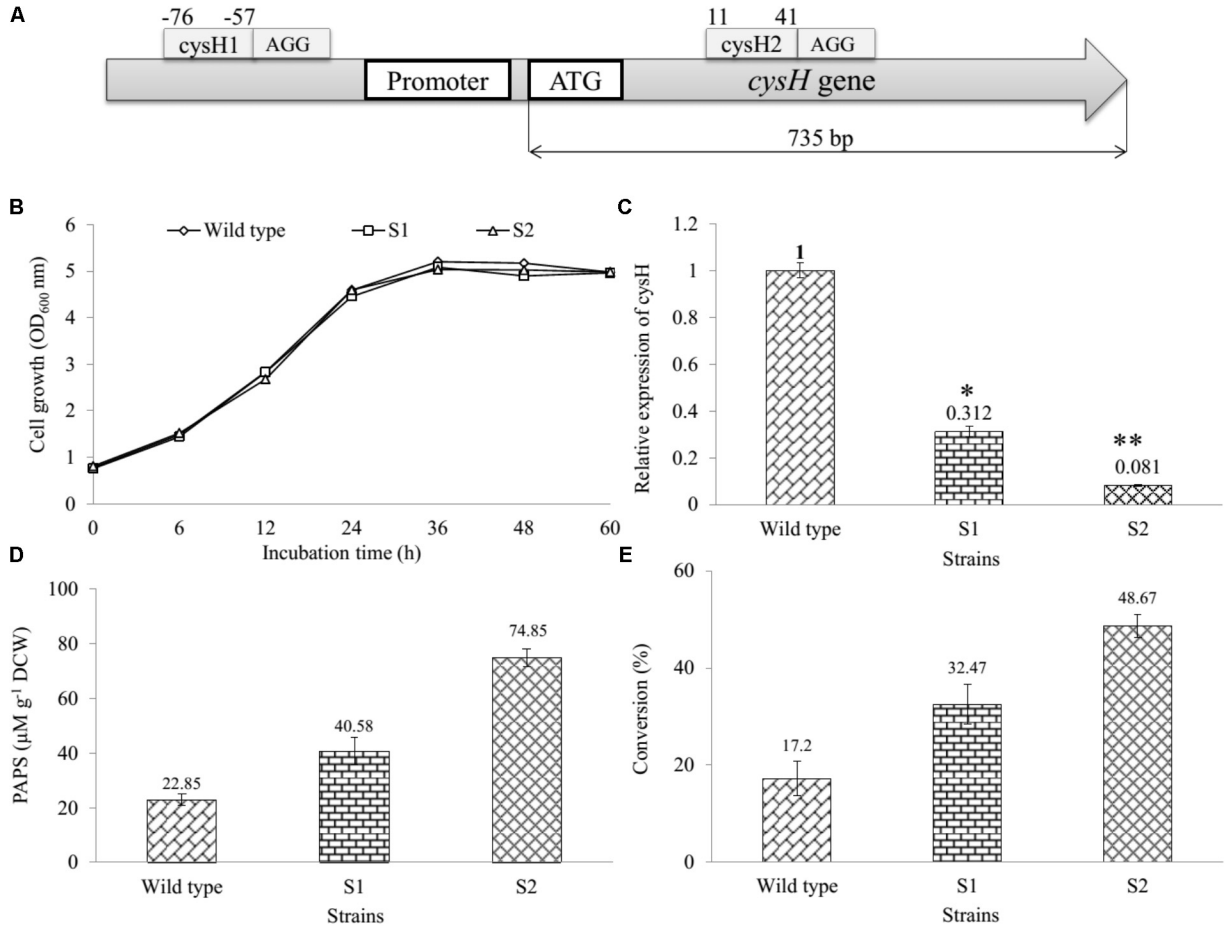

FIGURE 2 | Establishment and assessment of CRISPRi in sulfur metabolic pathway of *E. coli*. **(A)** The binding at different position on *cysH* gene using CRISPRi system. Sites selected *cysH1* (from position –76 to –57) after the PAM sequence AGG and *cysH2* (from position 11 to 44) after PAM sequence AGG. **(B)** Comparison of cell growth in LB broth medium at OD_{600}, **(C)** qRT-PCR analysis, **(D)** concentration of PAPS, and **(E)** the percentages of bioconversion between recombinant engineered *E. coli* carried out CRISPRi system targeting *cysH* gene and wild-type (*$p < 0.05$, **$p < 0.005$).

could be a good recombinant host system produce derivatives product form naringenin.

Regulating the Concentration of Inorganic Sulfate and Glucose in Medium

For regulation of the concentration $MgSO_4$, we used the M9 minimum media including the various concentrations of $MgSO_4$ (2, 5, 10, 15, and 20 mM) in comparison with the M9 medium without $MgSO_4$. The cell growth and substrate conversion were taken out at 12 h intervals. While the production was noticeably low in the medium without $MgSO_4$, the maximum bioconversion of substrate obtained 98.34% at 48 h with $OD_{600} \sim 9.94$ in M9 medium with 10 mM $MgSO_4$ when 100 μM naringenin was added (**Figures 3A,B**). Subsequently, the production was carried out by using an M9 medium with 10 mM $MgSO_4$ consisting of the various concentrations of glucose (2, 4, 6, 8, and 10%). The data showed that nearly 100% of 100 μM naringenin was converted to its sulfated derivatives during the addition 2, 4, and 6% glucose at 48 h with $OD_{600} \sim 9.94$, 10.73, and 9.57, respectively (**Figures 3C,D**). The M9 medium supplementation of 10 mM $MgSO_4$ and 2% glucose were selected for further optimizing the concentration of substrate.

Bioconversion With Different Naringenin Concentration and Scale-Up by Fermentation

Five various concentration of naringenin (200, 400, 600, 800, and 1,000 μM) were supplied for biocatalytic reaction system with the S2 strain. The OD_{600} and conversion rate of naringenin to its derivative were monitored at 12-h intervals. The highest production obtained 189 μM at 48 h with $OD_{600} \sim 10.24$ when 250 μM was fed under M9 medium including 10 mM $MgSO_4$ and 2% glucose (**Figures 4A,B**).

Finally, these optimal conditions were applied for the bioconversion process into the 3-L fermenter (Biotron, South Korea). When $OD_{600} \geq 6$, 0.1 mM IPTG was induced and the temperature decreased to 20°C. After 12 h induction, 250 μM (∼264 mg 3-L) of naringenin was fed to

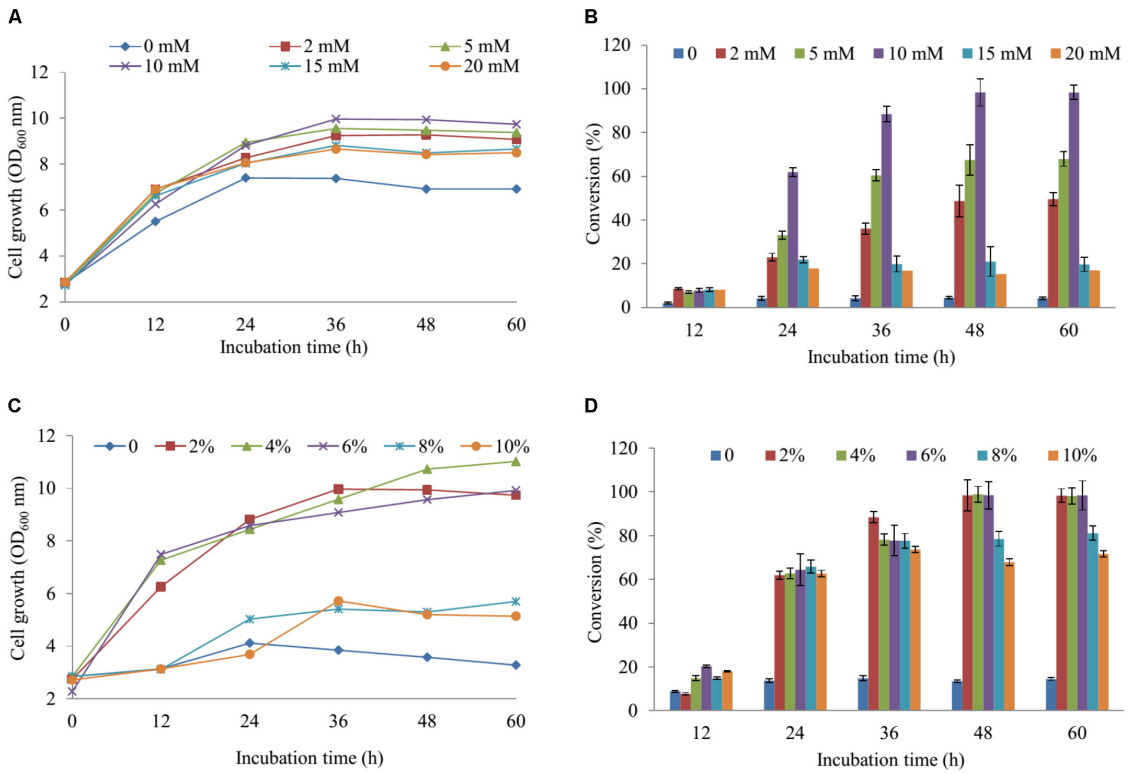

FIGURE 3 | The regulation of the concentration $MgSO_4$ and glucose in M9 media. The concentration of SO_4^{2-} effect on cell growth **(A)** and the production sulfated naringenin **(B)**. The percentages of glucose influent into cell growth **(C)** and bioconversion of naringenin to its derivative at the various incubation time **(D)**.

the culture under the pH and temperature were maintained at 7.4 and 30°C, respectively. The samples were taken at 12 h interval and measured by UFLC-PDA. UFLC-PDA analysis revealed that sulfated naringenin was obtained to 135.49 μM (∼143.1 mg 3-L) at 36 h with OD_{600} ∼ 51.06 (**Figure 4C**).

Structural Elucidation of Sulfated Naringenin

Previous study exhibited that even though no sulfating activity toward 5-, 3′and 4′-hydroxyflavone, At2g03770 showed the catalytic activity of 7-hydroxyflavone (Hashiguchi et al., 2013). We re-confirmed the structural compound by ^{1}H-NMR of naringenin standard and purified sulfated product at 300 MHz in DMSO-d_6. The ^{1}H-NMR spectrum of sulfated naringenin displayed the absence of OH− group signal at δ = 10.83 ppm for C-7 in comparison with the ^{1}H-NMR spectrum of naringenin (Supplementary Figure S10). Moreover, the ^{1}H-NMR spectrum of this compound exhibited a lower shift at H-6 and H-8 (Supplementary Table S4), indicating that the hydroxyl group at 7 positions of naringenin was substituted by a sulfate group. These data also agreed well with naringenin 7-sulfate obtained in the fungus *Cunninghamella elegans* NRRL 1392 (Ibrahim, 2000). Based ppm all the results, we could be identified that product was naringenin 7-sulfate. While naringenin was colorless compound, naringenin 7-sulfate was obtained as a yellowish solid (**Figure 4D**).

Antibacterial and Anticancer of Compounds

Results of disc diffusion assays displayed that naringenin showed only antibacterial activity against *Salmonella enterica* ATCC 14028 when 10 μL of 100 mM compound was used. In contrast, naringenin 7-sulfate exhibited broad-spectrum antibacterial activity against not only Gram-positive bacteria containing *Staphylococcus aureus* CCARM 0205, *S. aureus* CCARM 0204, *S. aureus* CCARM 3634, *Proteus hauseri* NBRC 3851, *Micrococcus luteus* KACC 13377, but also negative–positive bacteria consisting of *S. enterica* ATCC 14028, *Pseudomonas aeruginosa* KACC 10232, and *Enterobacter cloacae* subsp. *dissolvens* KACC 13002. Noticeably, naringenin 7-sulfate exhibited antibacterial activity against *M. luteus* KACC 13377 with a zone of inhibition values of 9.5 ± 0.18 mm. Moreover, we detected a zone of inhibition against *S. aureus* CCARM 0205 (MSSA) by naringenin 7-sulfate and ampicillin seems to share the most similarity with values of 7.0 ± 0.08 and 7.2 ± 0.09 mm. Interestingly, while naringenin and ampicillin did not show antibacterial activity against *P. aeruginosa* KACC 10232, naringenin 7-sulfate displayed a zone of inhibition values of 7.2 ± 0.12 mm (**Table 2** and Supplementary Figure S11). These results indicated that sulfation of naringenin at hydroxyl

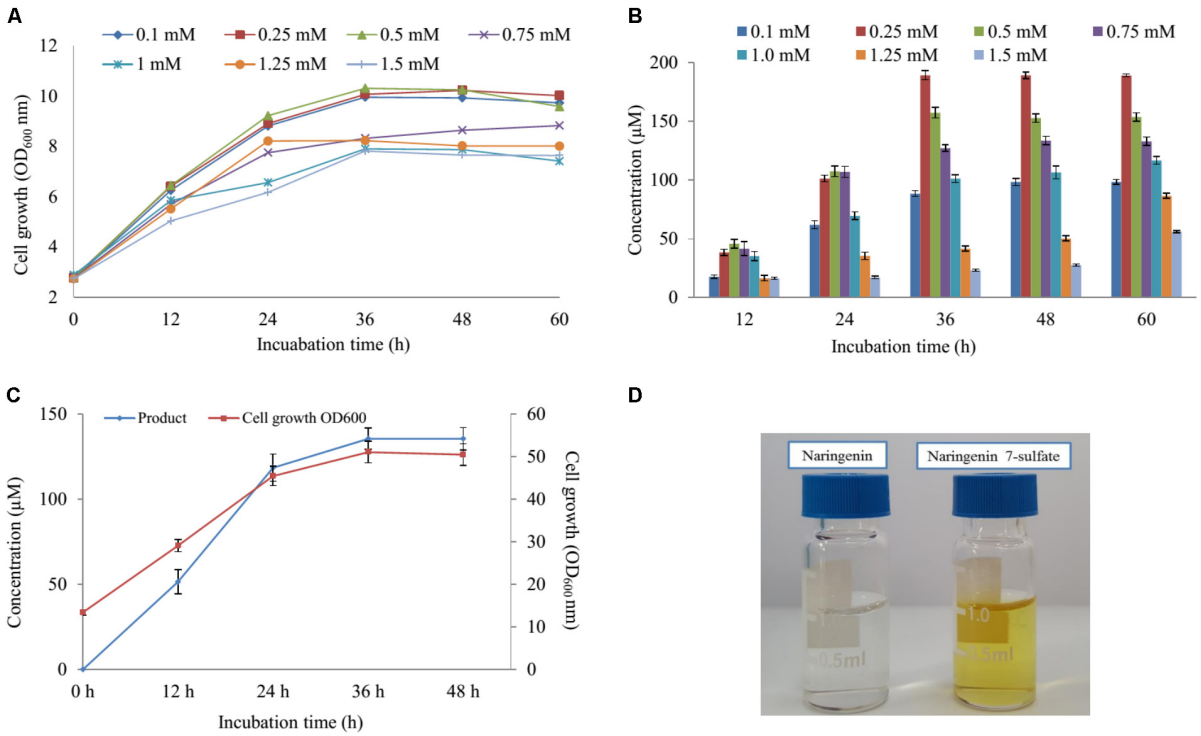

FIGURE 4 | The effect of substrate concentration and scale-up production. **(A)** Cell growth at $OD_{600\ nm}$ and **(B)** the substrate concentration optimization of naringenin in biotransformation. **(C)** Cell growth at $OD_{600\ nm}$ and the scale-up production sulfated naringenin in 3-L fermentation at different time intervals. **(D)** Comparison of color between substrate and product.

group of C-7 position could be advantageous for intensifying its antibacterial activity against various bacteria.

Furthermore, the cell viability data showed that naringenin 7-sulfate exhibited good anticancer activities where naringenin did not have anticancer activities against all cell lines. Cell viability of A375SM, MCF-7, AGS, and 267B1 treated 400 μM of naringenin 7-sulfate reduced approximately 48.85, 35.96, 41.78, and 71.20% ($p < 0.05$), respectively, in comparison with normal test (NT) (**Figure 5**). These results suggest that naringenin 7-sulfate was relatively less cytotoxic to non-cancer cell than cancer cell lines. In addition, the data showed naringenin 7-sulfate was less cytotoxic than naringenin in the non-cancer cell at 400 μM. As expected, taxol exhibited highly strong anticancer activity at nanomolar ranges of concentration. Taxol also inhibited A375SM and AGS cancer cell lines a little better than 267B1 non-cancer cell line with inhibition of 65.47, 63.40, and 49.90% at 100 nM, respectively (**Figure 5**). In conclusion, naringenin 7-sulfate could be the more potent anticancer agent with lower cytotoxicity against non-cancer cell than naringenin.

DISCUSSION

Sulfotransferases can be found in various life form from prokaryotes to eukaryotes (Suiko et al., 2017). In a model plant organism, *A. thaliana*, 18 different STs classified into seven groups are present (Klein and Papenbrock, 2004). Among all the *A. thaliana* STs, At2g03770 has been exploited as catalysis the sulfation for a variety of flavonoids (Hashiguchi et al., 2013). In this research, we produced 17.2 μM naringenin derivatives *via* overexpressed At2g03770 in *E. coli*. Although the yield of substantial amount of At2g03770 product appears in insoluble fraction might be led to the lower harvest of sulfated product (Supplementary Figure S2), the result indicated that heterologous expression STs from *A. thaliana* could be efficient produce sulfated compounds in whole cell biotransformations. Moreover, metabolic engineering in *E. coli* also might be applied for the enhanced production of prospective compounds extensively.

In *E. coli* sulfur metabolism, operon *cysUWAsbp* codes for a sulfate permease, an ATP binding cassette (ABC)-type transporter (Sekowska et al., 2000), carried the sulfate into the internal cell. Subsequently, ATP sulfurylase coded by operon *cysDN*, catalyzed an adenylylation of sulfate in the form of APS. After being activated by APS kinase *cysC*, APS is phosphorylated to PAPS (Hatzios and Bertozzi, 2011). PAPS is the universal sulfate donor in the reaction of sulfation performed by STs on the bacterial metabolite. On another hand, consumption of PAPS in sulfate assimilation pathway start catalyzed with the *cysH*-encoded PAPS ST. This reaction is continuously reduced for the biosynthesis of essential reduced sulfur metabolites (**Figure 1**). Therefore, CRISPRi has been introduced into background strain consist of expressed ST At2g03770, targeting strategy to inhibit PAPS consumption and subsequently increase

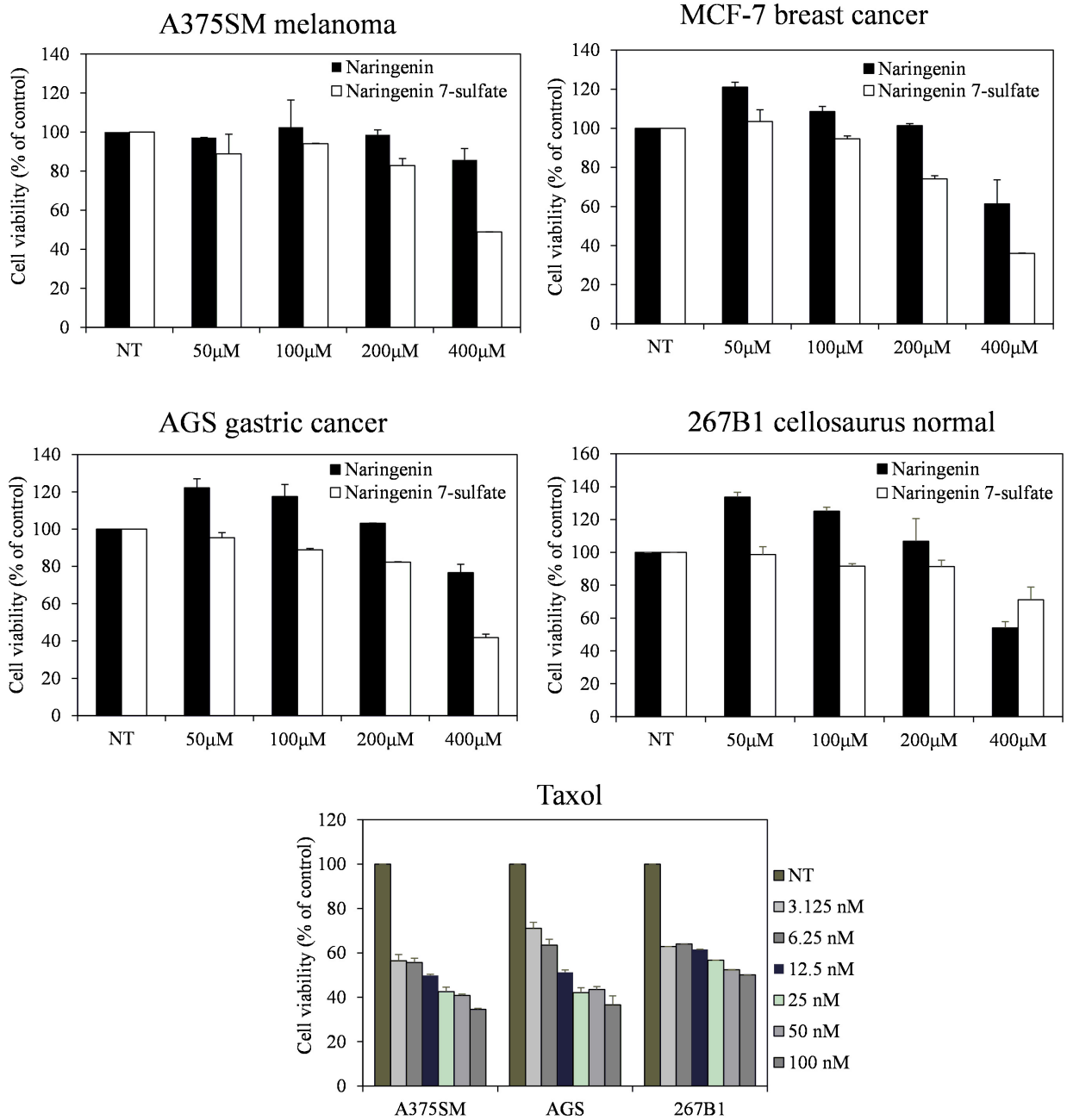

FIGURE 5 | Effect of naringenin and naringenin 7-sulfate on the cell viability of three cancer cell lines.

sulfated naringenin. This is the first time the production of sulfated flavonoids has published with the CRISPRi system in *E. coli*.

CRISPRi recently has applied to the enhanced biosynthesis of flavonoid (Cress et al., 2015) and *O*-methylated anthocyanin (Cress et al., 2017) through transcription regulation of metabolic pathway in *E. coli*. Compared to the traditional gene deletion methods, CRISPRi showed the great tool for targeted gene inhibition in microorganisms. Firstly, the construction of CRISPRi plasmid is simple and saving time because dCas9 protein and sgRNA expressed in one vector (Supplementary Figure S1). The efficiency of two different nucleotide sequence targeted on the *cysH* gene was not the same. The proportion suppression of spacer sequence bound to open reading frame region (*cysH2*) was 1.34-fold higher than the figure for spacer sequence bound to near promoter (*cysH1*), at 91.9 and 68.8%, respectively (**Figure 2C**). The reason behind the variation in gene expression level could be the corresponding to the distance of *cysH2* and *cysH1* to transcription start site, 10 bp of *cysH2* in compared with 56 bp of *cysH1* (**Figure 2A**). Moreover, the

regulation of sulfate metabolic pathway by CRISPRi system result in improving PAPS pool without affecting cell growth (**Figure 2B**). Ultimately, CRISPRi system not only reduces the metabolic burden associated with high-copy overexpression of heterologous ST At2g03770, but also increase production of target molecules. In the whole cell biocatalysis system, production of naringenin derivatives increased by up to 2.83-fold (**Figure 2E**). This result suggested that S2 strain not only led to the accumulation of intracellular PAPS but also improved the efficiency of sulfation of naringenin.

Recently, the analysis of polar molecules such as ATP, ADP, and PAPS was carried out *via* liquid chromatography-mass spectrometry (Johnsen et al., 2011; Dowood et al., 2016). In this study, UHPLC-PDA coupled HR-QTOF ESI/MS allowed the detection PAPS, although the quantity of PAPS was lowed in the cytoplasm of *E. coli*. The method not only is high accuracy but also simple to perform with general solvent and saving time. Moreover, this method was very significant for the separation between PAPS and nucleoside triphosphates as ATP and ADP, because these compounds were the same physical properties (Dowood et al., 2016).

The cultivation of the wild-type and S1, S2 strains was the initial phase of rapid cell growth at 37°C, followed by a growth arrest phase at 20°C, and the biosynthesized compound produced at 28°C. The growth-arrested *E. coli* induced by using low temperature led to improved the conformational quality and the solubility of STs from *A. thaliana* and CRISPRi systems (Vera et al., 2007; Cress et al., 2015), which associated with an increased sustained viability and an extended production phase (Rosano and Ceccarelli, 2014). This experiment has a significant impact on the optimal production of the target compound. We decided to the production sulfated naringenin in M9 medium containing $MgSO_4$ and glucose as sulfur and carbon sources, respectively. Almost 100% of conversion rate from a substrate to its derivative was obtained in media consisting of 10 mM $MgSO_4$ and 2–6% glucose, indicating that sulfated compound all accumulated in the extracellular fraction. One possible reason behind this result could be organic anion molecules as sulfated naringenin has been eliminated into extracellular by *E. coli* multidrug resistance ATP binding cassette transporters (Chang and Roth, 2001). On the other hand, sulfated naringenin including the negative charged SO_4^{2-} led to might not to cross the cell membrane of *E. coli*. Furthermore, even though the media lacking $MgSO_4$ resulted in the cell growth was low, the production still obtained around 4.42% (**Figures 3A,B**). The reason could be because of when the growth medium absent inorganic sulfur, *E. coli* can induce a series of sulfate starvation-inducible genes led to utilize organosulfur compound as a source of sulfur (van der Ploeg et al., 2001). Moreover, the media absence glucose also caused the production was low as well as the cell growth inefficient (**Figures 3C**, **4D**). These results demonstrated that both SO_4^{2-} and glucose are an essential factor produce sulfated naringenin by At2g03770-expressed in *E. coli* cells. However, the high concentration of SO_4^{2-} (above 15 mM) and glucose (over 6%), as well as more than 0.5 mM of the substrate, could be a reason for inhibition of sulfation product in *E. coli* whole cell (**Figures 3**, **4**). Finally, the engineered *E. coli* S2 strain has been applied successfully for the large-scale production of naringenin 7-sulfate, which obtained at 135.49 μM [∼143.1 mg (47.7 mg L^{-1})] in a 3-L fermenter (**Figure 4C**). These results indicate that the system is efficient and could be applied for other flavonoids to generate the libraries of molecules with various sulfation approaches.

We also evaluated the antibacterial activity of naringenin and its derivative. Five Gram-positive bacteria *S. aureus* CCARM 0205, *S. aureus* CCARM 0204, *S. aureus* CCARM 3634, *P. hauseri* NBRC 3851, *M. luteus* KACC 13377 and three Gram-negative bacteria *S. enterica*, *P. aeruginosa*, *E. cloacae* were sensitive with naringenin 7-sulfate. This result indicated that negatively charged sulfate group might be improved to naringenin for antibacterial activity, however, the mechanism of this compound against bacterial pathogens have been not reported. In addition, naringenin derivative exhibited the most potential anticancer activity against three tested cancer cell lines. The compound showed the similarity in features between its and taxol the less cytotoxic to non-cancer cell line in comparison with cancer cell lines (**Figure 5**). This is the first report of the activity of naringenin 7-sulfate against A375SM, MCF-7 and AGS cancer cell lines. The previous research has shown that the substituent at the C-7 position of naringenin containing thiophenecarboxylate, phenyl carbonate, isobutyrate, methyl benzoate, and allyloxy inhibited effects on HCT116 human colon cancer cell line *via* block G1 cell cycle progression by interaction with cyclin-dependent kinase 2 (CDK2) (Yoon et al., 2013). This possible mechanism behind the anticancer activity of naringenin 7-sulfate against three tested cancer cell lines, however, the exact mechanisms of action of this compound must confirm in further studies.

In summary, we targeted to improving production of sulfated flavonoids in engineered *E. coli*. This is the first report on CRISPRi mediated inhibition in the sulfur metabolism of *E. coli*. Repression of key reduced sulfur metabolite enzyme *cysH* by over 91%, causing increase intracellular PAPS accumulation and enhancement of naringenin 7-sulfate over 3.28- and 2.83-fold compared to control, respectively. Further media culture optimization led to obtained at 135.49 μM [∼143.1 mg (47.7 mg L^{-1})] in a 3-L fermenter. This engineered *E. coli* opened prospects for the biosynthesis of the sulfated flavonoids. In addition, naringenin 7-sulfate exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria. This compound could be also used as a cancer drug when shown to anticancer activity against A375SM melanoma, MCF-7 breast, and AGS gastric cancer cell lines.

AUTHOR CONTRIBUTIONS

LC designed, performed the majority of the experiment work, analyzed data, and wrote the manuscript. DD and TY helped in analyzing data. HS and HJ did the majority of anticancer activities. JS and LC were responsible for the original concept and supervised the work. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01671/full#supplementary-material

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Enhancing Production of Pinene in *Escherichia coli* by Using a Combination of Tolerance, Evolution, and Modular Co-culture Engineering

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α-Pinene is a natural and active monoterpene, which is widely used as a flavoring agent and in fragrances, pharmaceuticals, and biofuels. Although it has been successfully produced by genetically engineered microorganisms, the production level of pinene is much lower than that of hemiterpene (isoprene) and sesquiterpenes (farnesene) to date. We first improved pinene tolerance to 2.0% and pinene production by adaptive laboratory evolution after atmospheric and room temperature plasma (ARTP) mutagenesis and overexpression of the efflux pump to obtain the pinene tolerant strain *Escherichia coli* YZFP, which is resistant to fosmidomycin. Through error-prone PCR and DNA shuffling, we isolated an *Abies grandis* geranyl pyrophosphate synthase variant that outperformed the wild-type enzyme. To balance the expression of multiple genes, a tunable intergenic region (TIGR) was inserted between *A. grandis* $GPPS^{D90G/L175P}$ and *Pinus taeda* $Pt1^{Q457L}$. In an effort to improve the production, an *E. coli-E. coli* modular co-culture system was engineered to modularize the heterologous mevalonate (MEV) pathway and the TIGR-mediated gene cluster of *A. grandis* $GPPS^{D90G/L175P}$ and *P. taeda* $Pt1^{Q457L}$. Specifically, the MEV pathway and the TIGR-mediated gene cluster were integrated into the chromosome of the pinene tolerance strain *E. coli* YZFP and then evolved to a higher gene copy number by chemically induced chromosomal evolution, respectively. The best *E. coli-E. coli* co-culture system of fermentation was found to improve pinene production by 1.9-fold compared to the mono-culture approach. The *E. coli-E. coli* modular co-culture system of whole-cell biocatalysis further improved pinene production to 166.5 mg/L.

Keywords: pinene biosynthesis, *Escherichia coli,* tolerance engineering, directed evolution, chemically induced chromosomal evolution, modular co-culture

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INTRODUCTION

α-Pinene is a natural and active monoterpene, which is widely used in flavorings, fragrances, insecticides, pharmaceuticals, and fine chemicals (Breitmaier, 2006; Behr and Johnen, 2009; Kirby and Keasling, 2009; Gandini and Lacerda, 2015). It was recently produced as a candidate renewable jet fuel due to its favorable energy content, cold weather properties, and high octane/cetane

numbers (George et al., 2015). The main source of pinene is turpentine, a by-product of the wood pulp industry (Behr and Johnen, 2009). However, this extraction from plants is tedious and inefficient and requires substantial expenditure of natural resources due to low content (Chang and Keasling, 2006). Therefore, there is much interest in developing biotechnologies for pinene production from renewable resources by engineering microorganisms. Similar to other monoterpenes, α-pinenes are biosynthesized from the C5 intermediates isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) via geranyl diphosphate synthase (GPPS). The head-to-tail condensation produces geranyl diphosphate (GPP, C10), which is, in turn, cyclized by pinene synthase (PS) to produce either α- or β-pinene. *Escherichia coli* (Yang et al., 2013; Sarria et al., 2014; Tashiro et al., 2016) and *Corynebacterium glutamicum* (Kang et al., 2014) have been engineered to produce α-pinene. α-Pinene (5.4 mg/L) has been produced in engineered *E. coli* through the introduction of a heterologous mevalonate (MEV) pathway and α-pinene synthase (Pt30) from *Pinus taeda* (Yang et al., 2013). The combinatorial expression of *Abies grandis* GGPS-PS fusion proteins enhanced pinene production (32 mg/L) in *E. coli* (Sarria et al., 2014). The directed evolution of α-pinene synthase (Pt1) from *P. taeda* increased α-pinene productivity. *E. coli* plasmid-expressing the evolved α-pinene synthase ($Pt1^{Q457L}$) from *P. taeda*, MEV pathway enzymes, IPP isomerase and *A. grandis* GGPS produced the highest levels of pinene (140 mg/L) in a flask culture to date (Tashiro et al., 2016). The coexpression of native 1-deoxy-d-xylulose-5-phosphate synthase (Dxs) and isopentenyl diphosphate isomerase (Idi) with *P. taeda* PS and *A. grandis* GPPS in *C. glutamicum* yielded a pinene level of 27 μg/g cell dry weight (Kang et al., 2014).

However, the production level of pinene is much lower than that of hemiterpene (isoprene) (Whited et al., 2010) and sesquiterpenes (farnesene) (Zhu et al., 2014) to date. Pinene is highly toxic to *E. coli*. *E. coli* growth is inhibited by 0.5% pinene (Dunlop et al., 2011). The inherent tolerance of *E. coli* may limit the production potential. It was demonstrated that increasing the tolerance of *E. coli* by overexpressing the efflux pump AcrBDFa (YP_692684) from *Alcanivorax borkumensis* significantly enhanced limonene production (Dunlop et al., 2011). Another reason for the lower yield may be that PS has a lower expression level and/or lower enzymatic activity in *E. coli*. Thus, we first combined tolerance engineering with directed evolution of the enzyme to improve pinene production in *E. coli*.

Recently, there has emerged a new modular co-culture engineering approach for engineering microorganisms. Modular co-culture engineering approaches divide a complete biosynthetic pathway into separate serial modules, which are introduced into different strains to accommodate individual modules for achieving designed biosynthesis (Zhang and Wang, 2016). The advantages of using modular co-culture engineering include the following: (1) reducing the metabolic burden on each host strain; (2) providing diversified cellular environments for functional expression of the different pathway genes; (3) reducing the undesired interference of different pathways; (4) easily balancing the biosynthetic pathway between individual pathway modules by simply changing the strain-to-strain ratio; (5) high-efficiency utilization of complex materials containing multiple active substrates; and (6) supporting the plug-and-play biosynthesis of various target products (Zhang and Wang, 2016). Thus, modular co-culture engineering was also used to enhance pinene production in *E. coli*.

MATERIALS AND METHODS

Strains, Plasmids, and Primers

The bacterial strains and plasmids used in this study are listed in **Table 1**. The primers used in this study are listed in Supplementary Table 1.

Genetic Methods

pMEVI was derived from pJBEI-6409 (Alonso-Gutierrez et al., 2013), which was obtained from Addgene. pJBEI-6409 contains six genes of the MEV pathway (*atoB* from *E. coli*, *HMGS,* and *HMGR* from *Staphylococcus aureus* and *MK*, *PMK,* and *PMD* from *Saccharomyces cerevisiae*, *idi* from *E. coli*, *GPPS* from *A. grandis,* and limonene synthase gene (*LS*) from *Mentha spicata*). The *GPPS-LS* gene cluster was removed from pJBEI-6409 to obtain pMEVI. The fusion gene cluster of the codon-optimized *GPPS* and *PS* from *A. grandis* with a $(GSG)_2$ linker was synthesized by Suzhou GENEWIZ, Inc. (Suzhou, China) and ligated into pQE30 to obtain pQE-GPPS-L-PS. The GPPS-PS gene cluster from pQE-GPPS-L-PS was inserted into the BamHI/XhoI sites of pMEVI to obtain pMEVIGPS. The evolved codon-optimized *Pt1* ($Pt1^{Q457L}$) from *P. taeda* was synthesized by Suzhou GENEWIZ, Inc. (Suzhou, China) and ligated into pQE30 to obtain pQE-$Pt1^{Q457L}$. The *PS* gene of pQE-GPPS-L-$PS^{DNA\ shuffling}$ was replaced with the $Pt1^{Q457L}$ gene to obtain pQE-$GPPS^{MUT}$-L-$Pt1^{Q457L}$.

The *acrB* and *acrAB* were amplified from *E. coli* and inserted into pZEABP to obtain pZEA-acrB and pZEA-acrAB, respectively. The *P. putida* KT2440 *ttgB*, *P. putida* KT244 *mexF,* and *A. borkumensis acrBDFa* were amplified from pBbA5K-EPL11, pBbA5K-EPL14, and pBbA5K-EPL95 and inserted into pZEABP to obtain pZEA-ttgB, pZEA-mexF, and pZEA-acrBDFa, respectively.

The TIGR-mediated $GPPS^{MUT}$-$Pt1^{Q457L}$ gene cluster was cut from pQE-$GPPS^{MUT}$-TIGR-$Pt1^{Q457L}$ with EcoRI/HindIII and then cloned into EcoRI/HindIII-digested pHKKF3T5b to obtain pHKKF3T5b-$GPPS^{MUT}$-TIGR-$Pt1^{Q457L}$. The MEVI operon was cut from pMEVI with EcoRI/XhoI and then cloned into EcoRI/SalI-digested pP21KF3T5b to obtain pP21KF3T5b-MEVI. Chromosomal integration was carried out by direct transformation as described by Chen et al. (2013). Chemically induced chromosomal evolution (CIChE) of the above construct was carried out by subculturing the resulting strains in 5 mL Super Optimal Broth (SOB) medium with increasing concentrations of triclosan in 15 mL culture tubes, as described by Chen et al. (2013). The strains were grown to the stationary phase in 1 μM triclosan for pP21KF3T5b-$GPPS^{MUT}$-TIGR-$Pt1^{Q457L}$ or 0.25 μM for pHKKF3T5b-MEVI. Fifty milliliters of the culture were subcultured into a new culture tube, in

TABLE 1 | Strains and plasmids used in this study.

Name	Description	Reference/Sources
STRAIN		
E. coli BW25113	*lacI*^{q}*rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$*hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Datsenko and Wanner, 2000
E. coli BW25113 (P_{T5}-dxs)	*E. coli* BW25113, P_{dxs}::P_{T5}	Weng et al., 2012
E. coli YZ-3	The ALE strain from *E. coli* BW25113 (P_{T5}-dxs), tolerance to 2.0% pinene	This study
E. coli YZ-3-A	*E. coli* YZ-3, P_{acrAB}::P37	This study
E. coli YZ-3-A-T	*Pseudomonas putida* KT2440 *ttgB* under the control of P37 promoter was integrated into the chromosome of *E. coli* YZ-3-A	This study
E. coli YZFP	Pinene tolerance strain, *E. coli* YZ-3-A-T mutant resistant to fosmidomycin	This study
E. coli PINE	Pinene producer, ClChE strain from *E. coli* YZFP after integration of the TIGR-mediated gene cluster of the *A. grandis* GPPS^{Mut}-*P. taeda* Pt1^{MUT} gene cluster	This study
E. coli MEVI	ClChE strain from *E. coli* YZFP after integration of the mevalonate pathway	This study
PLASMID		
pJBEI-6409	Addgene plasmid #47048, pBbA5c-MTSAe-T1f-MBI(f)-T1002i-Ptrc-trGPPS(co)-LS) coding for MEV pathway enzymes to produce limonene from glucose in *E. coli*, p15A *ori,* P_{lacUV5} promoter, cm^{r}	Alonso-Gutierrez et al., 2013
pMEVI	pBbA5c-MTSAe-T1f-MBI(f)-T1002i coding for MEV pathway enzymes and *E. coli* Idi, p15A *ori,* P_{lacUV5} promoter, cm^{r}	This study
pMEVIGPS	pBbA5c-MTSAe-T1f-MBI(f)-T1002i-trGPPS$_{A.grandis}$-PS$_{A.grandi}$ coding for MEV pathway enzymes to produce pinene from glucose in *E. coli,* p15A *ori,* P_{lacUV5} promoter, cm^{r}	This study
pBbA5K-EPL11	Addgene plasmid #45403, pBbA5K containing *Pseudomonas putida* KT2440 *ttgB*	Dunlop et al., 2011
pBbA5K-EPL14	Addgene plasmid #45405, pBbA5K containing *P. putida* KT2440 *mexF*	Dunlop et al., 2011
pBbA5K-EPL95	Addgene plasmid #45434, pBbA5K containing *Alcanivorax borkumensis acrBDFa*	Dunlop et al., 2011
pZEABP	Constitute expression vector, pBR322 *ori*, P37 promoter, Amp^{r}, BglBrick, ePathBrick containing four isocaudamer (AvrII, NheI, SpeI, and XbaI)	Li et al., 2015
pZEA-acrB	pZEA*BP containing *E. coli acrB,* pBR322 *ori*, P37 promoter, Amp^{r}	This study
pZEA-acrAB	pZEA*BP containing *E. coli acrAB,* pBR322 *ori*, P37 promoter, Amp^{r}	This study
pZEA-mexF	pZEA*BP containing *P. putida* KT2440 *mexF,* pBR322 *ori*, P37 promoter, Amp^{r}	This study
pZEA-acrBDFa	pZEA*BP containing *A. borkumensis acrBDFa,* pBR322 *ori*, P37 promoter, Amp^{r}	This study
pZEA-ttgB	pZEA*BP containing *P. putida* KT2440 *ttgB,* pBR322 *ori*, P37 promoter, Amp^{r}	This study
pQE30	*E. coli* expression vector, T5 promoter, pBR322 *ori*, Am^{r}	Invitrogen
pQE-GPPS-L-PS	pQE30 harboring the fusion gene of the codon-optimized *A. grandis* GPPS and PS with a $(GSG)_2$ linker	This study
pQE-GPPS$_{6AA}$-L-PS	pQE30 harboring the fusion gene of the 6AA method optimized *A. grandis* GPPS and PS with a $(GSG)_2$ linker	This study
pQE-GPPS-L-PS^{epPCR}	pQE30 harboring the evolved fusion gene of the 6AA method optimized *A. grandis* GPPS and PS with a $(GSG)_2$ linker after error-prone PCR	This study
pQE-GPPS-L-PS$^{DNA\ shuffling}$	pQE30 harboring the evolved fusion gene of the 6AA method optimized *A. grandis* GPPS and PS with a $(GSG)_2$ linker after error-prone PCR and DNA shuffling	This study
pQE-GPPS^{MUT}-L-Pt1^{Q457L}	pQE30 harboring the fusion gene of the evolved *A. grandis* GPPS and *P. taeda* Pt1^{Q457L} with a $(GSG)_2$ linker	This study
pQE-GPPS^{MUT}-Pt1 ^{Q457L}	pQE30 harboring *A. grandis* GPPS$^{D90G/L175P}$ and *P. taeda* Pt1^{Q457L}	This study
pQE-GPPS^{MUT}-TIGR-Pt1^{Q457L}	pQE30 harboring the TIGR-mediated gene cluster of the evolved *A. grandis* GPPS and *P. taeda* Pt1^{Q457L}	This study
pP$_{rstA}$-GFP	the IPP/FPP sensor plasmid, pZSBP derivative with GFP, P_{rstA} promoter, kan^{r}	Shen et al., 2016
pP21KF3T5b	ClChE integration expression vector, *attP*$_{P21}$ site, kan^{r}	Chen et al., 2013
pHKKF3T5b	ClChE integration expression vector, *attP*$_{HK}$ site, kan^{r}	Chen et al., 2013
pHKKF3T5b-GPPS^{MUT}-TIGR-Pt1^{Q457L}	pHKKF3T5b harboring the TIGR-mediated gene cluster of the evolved *A. grandis* GPPS and *P. taeda* Pt1^{Q457L}	This study
pP21KF3T5b-MEVI	pP21KF3T5b harboring MEV pathway enzymes and *E. coli* Idi	This study
pCas	*E. coli* cas9 expression vector	Jiang et al., 2015
pCas*	*E. coli* cas9 (K848A/K1003A/ R1060A) expression vector	This study
pTargetF	*E. coli* sgRNA expression vector	Jiang et al., 2015
pTargetB	*E. coli* sgRNA expression vector, BglBrick vector	This study

which the triclosan concentration was doubled to 132 or 32 μM and allowed to grow to the stationary phase. The process was repeated until the desired concentration was reached. The *recA* gene of the CIChE strain was then deleted by the markerless deletion approach using the isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible *ccdB* as a counter-selectable marker (Wei et al., 2016).

Gene replacement of the native promoter of *E. coli acrAB* and the integration of *ttgB* from *P. putida* KT2440 were carried out by the CRISPR-Cas method as described by Jiang et al. (2015). To enhance specificity and reduce off-target effects, the *cas9* on pCas (Jiang et al., 2015) was site-directed mutated into *cas9(K848A/K1003A/R1060A)* as described as Slaymaker et al. (2016) to obtain pCas*. To easily assemble the sgRNA sequence using the BglBrick standard method, the BglII site in the sgRNA plasmid pTargetF was first removed, and then a BglII site was added in the front of EcoRI site to obtain the sgRNA plasmid pTargetB.

Adaptive Laboratory Evolution for Improving Pinene Tolerance

A 1-mL culture of logarithmic phase *E. coli* was collected by centrifugation, washed twice with saline, and diluted to a cell concentration of 10^6 to 10^7 with physiological saline. Then, atmospheric and room temperature plasma (ARTP) mutagenesis was performed using an ARTP mutation system (ARTP-IIS, Tmaxtree Biotechnology Co, Ltd, Wuxi, China) with the following parameters: (1) the radio frequency power input was 100 W; (2) the flow of pure helium was 10 standard liters per min; (3) the distance between the plasma torch nozzle exit and the slide was 2 mm; and (4) the different treatment times were selected (10, 20, 40, 60, 80, 100, and 120 s). Ten microliters of the aforementioned cell dilution were evenly scattered on the slide and subjected to ARTP mutagenesis. After treatment, the slide was washed with LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl), transferred to 5 mL of LB medium with 0.5% pinene in a 15 mL falcon tube, and cultivated at 30 °C and 200 rpm for 24 h. The cultures were serially passed into fresh medium (initial OD_{600} of 0.2) daily. Continuously repeating this transfer procedure at 0.5% pinene until OD_{600} at 24 h did not increase further, the culture was then sequentially transferred to a pinene concentration of 1.0%, 1.5% and 2.0%. The cultures were frozen and stored at −80°C at every pinene concentration.

The cultures of 2.0% pinene stored at −80°C were transferred by the IPP/FPP sensor plasmid pP_{rstA}-GFP. Single colonies were inoculated in individual wells of a 48 deep-well microplate (4.6 mL) containing 600 μL of LB medium and incubated at 30°C and 200 rpm for 24 h on a Multitron shaker (Infors). The cells were harvested by centrifugation at 14000 × g for 2 min and then resuspended with 0.6 mL (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). Then, 200 μL of the bacterial culture was transferred into a 96-well plate in which the OD_{600} and fluorescence were read with the excitation at 485 nm and emission at 528 nm using a SynergyNeo2 multi-mode reader (SynergyNeo2, BioTek, USA).

Generating Random Mutagenesis Libraries Using Error-Prone PCR and Screening

The random mutagenesis libraries of the fusion gene cluster of *AgGPPS-AgPS* after optimization of the first 18 codons using the 6AA method (Boë et al., 2016) were constructed through error-prone PCR. The gene cluster of *AgGPPS-AgPS* was amplified from pQE-$GPPS_{6AA}$-L-PS using the primers EcoRI-GPPS/HindIII-PS. The error-prone PCR reaction mixture consisted of 5 mM $MgCl_2$, 0.3 mM $MnCl_2$, 0.2 mM each of dATP and dGTP, 1 mM each of dCTP and dTTP and Tag DNA polymerase. The PCR product was digested by EcoRI/HindIII, ligated into the EcoRI/HindIII sites of pQE30, and then transferred into the lycopene-producing strain *E. coli* LYCOP to generate the mutant library.

The mutant library was plated on LB agar with ampicillin and IPTG. The plates were incubated at 30°C overnight. The mutant plasmid was isolated from the whiter colony and then transferred into component *E. coli* BW25113 (P_{T5}-dxs, pMEVI). The pinene productions of them were analyzed in a shake flask.

Generating Random Mutagenesis Libraries Using DNA Shuffling and Screening

DNA shuffling experiments were performed by the following steps: parental template preparation, DNase I digestion, primer-less PCR and PCR with primers. The mutant plasmids from the 7 colonies resulted from error-prone PCR and were used as the template to amplify the gene cluster fragments with the primers EcoRI-GPPS/HindIII-PS. Following purification, 2 μg of the eight PCR products was mixed and treated with 0.02 U of DNaseI in 100 μL of the 10 × DNaseI buffer on ice for 2 min and terminated by the loading buffer containing SDS. The purified fragments of 50–300 bp were used in the primer-less PCR reactions to reassemble into full-length genes. The primer-less PCR reaction mixture contained 0.5 mM each dNTP, 10 × Taq buffer and 0.5 μL Taq DNA polymerase (Takara). The PCR reaction conditions were as follows: 95°C for 1 min, 35 cycles of 94°C for 30 s, 45°C for 30 s, 72°C for 3 min, and final incubation at 72°C for 8 min. The PCR products with the correct size were purified and subjected to PCR amplification using the same conditions with the primers EcoRI-GPPS/HindIII-PS. Finally, the mutated PCR products of the full-length gene were digested by EcoRI/HindIII, ligated into the EcoRI/HindIII sites of pQE30, and transferred into the lycopene-producing strain *E. coli* LYCOP to generate the mutant library.

The mutant library was plated on LB agar with ampicillin and IPTG. The plates were incubated at 30°C overnight. Single colonies with a whiter color were inoculated in individual wells of a 48 deep-well microplate (4.6 mL) containing 1 mL of LB medium and incubated at 30°C and 200 rpm on a Multitron shaker (Infors). After 8 h, the cultures were induced with 1 mM IPTG and overlaid with 20% dodecane to trap pinene. After induction, the cultures were incubated at 30°C and 200 rpm for 48 h. The pinene concentration in individual wells was assayed using the concentrated sulfuric acid method as follows. One hundred microliters of the dodecane layer were mixed with 200 μL sulfuric acid, then inoculated for 5 min in

boiling water, and the absorbance of the reaction solution at 450 nm was determined using a spectrophotometer (Shimadzu, Japan).

Creating TIGR Libraries and Screening

TIGRs were synthesized using PCR to assemble the oligonucleotides into chimeric DNA sequences as described by Pfleger et al. (2006) and Li et al. (2015). Briefly, 40 mmols of an equimolar oligonucleotide (A, B, C, and D in Supplemental Table 1) mixture was added to a mixture containing 2.5 units of Primer Star DNA Polymerase (Takara, Dalian, China). The assembly was conducted over 35 cycles of PCR for 10 s at 98°C, 30 s at 72°C, and 20 + 5 s/cycle at 72°C. The assembly products were purified using a nucleotide removal column and amplified using the end-specific primers TIGRs-F(X)/TIGRs-R(A) and then cloned into the SacI/SalI sites of pQE-GPPS^{MUT}-Pt1 ^{Q457L} to obtain the plasmid libraries pQE-GPPS^{MUT}-TIGRs-Pt1^{Q457L}. The plasmid libraries were transferred into component *E. coli* BW25113 (P_{T5}-dxs, pMEVI) to generate the mutant library.

The TIGR library was plated on LB agar with ampicillin. The plates were incubated at 30°C overnight. Single colonies were inoculated in individual wells of a 48 deep-well microplate (4.6 mL) containing 1 mL of LB medium and incubated at 30°C and 200 rpm on a Multitron shaker (Infors). After 8 h, the cultures were induced with 1 mM IPTG and overlaid with 20% dodecane to trap pinene. After induction, the cultures were incubated at 30°C and 200 rpm for 48 h. The pinene concentration in individual wells was assayed using the above concentrated sulfuric acid method.

Pinene Biosynthesis in Shake Flasks

For pinene fermentation production, a single colony was inoculated into 5 mL of LB medium in a falcon tube, which was cultured overnight at 37°C. The overnight seed culture was then inoculated into 50 mL of SBMSN medium with a starting OD_{600} of 0.1. SBMSN medium (pH 7.0) containing the following (g/L): sucrose 20, peptone 12, yeast extract 24, KH_2PO_4 1.7, K_2HPO_4 211.42, $MgCl_2{\cdot}6H_2O$ 1, ammonium oxalate 1.42, and Tween-80 2. The main cultures were then incubated at 37°C and 200 rpm until an OD_{600} of 0.8 was reached. Then, the cultures were induced with 1 mM IPTG and overlaid with 20% dodecane to trap pinene. After induction, the cultures were incubated at 30°C and 130 rpm for 72 h.

Co-culture of *E. coli* PINE and MEVI for Pinene Production

E. coli PINE and MEVI cells were first separately grown in 5 mL SBMSN medium in a falcon tube at 37°C overnight. The overnight culture was inoculated into 50 mL of SBMSN medium with a starting OD_{600} of 0.1 and incubated at 37°C and 200 rpm until an OD_{600} of approximately 6.0 was reached. The cultures were then incubated at 20°C and 200 rpm for 16 h. For pinene biosynthesis using co-cultures, the *E. coli* PINE culture and the desired amount of the *E. coli* MEVI culture were inoculated into the 30 mL SBMSN medium with a starting OD_{600} of 0.1. The mixed culture was culture at 37°C and 200 rpm until an OD_{600} of 0.8 was reached. Then, the cultures were overlaid with 20% dodecane to trap pinene, and were incubated at 30°C and 130 rpm for 72 h.

Whole-Cell Biocatalysis for Pinene Production

A single colony of *E. coli* PINE and MEVI was separately inoculated into 5 mL of SBMSN medium in a falcon tube, which was cultured overnight at 37°C. The overnight cultures were then inoculated into 50 mL SBMSN medium with a starting OD_{600} of 0.1. The cultures were then incubated at 37°C and 200 rpm until an OD_{600} of approximately 6.0 was reached. Then, the cultures were incubated at 20°C and 200 rpm for 16 h. Finally, the *E. coli* PINE culture was mixed with the *E. coli* MEVI culture at the inoculation ratio of 2:1. The mixed cells were harvested by centrifugation (6000 × g at 4°C) and washed twice with cooled phosphate buffer (0.1 M, pH 7.0).

For biocatalysis, the above cells were resuspended in 10 mL phosphate buffer (0.1 M, pH 7.0) containing 20 g/L of sucrose, 10 mM $MgCl_2$ and 5 mM $MnCl_2$ to form the cell suspension (OD_{600} = 30). The reaction mixture was overlaid with 20% dodecane. The catalysis was performed for 28 h at 30°C and 130 rpm.

GC Analysis

Five hundred microliters of the dodecane layer was placed in a 1.5-mL microcentrifuge tube and centrifuged at 25,000 × g for 1 min, and 50 μL of dodecane was diluted in 450 μL of ethyl acetate spiked with the internal standard limonene (10 μg/L). The samples were analyzed by GC-FID by using a standard curve of α-pinene (Sigma Aldrich). The GC-FID (Techcomp GC7900, Techcomp Ltd, China) was used with a TM-5 column (30 m × 0.32 mm × 0.50 μm). The inlet temperature was set to 300°C, with the flow at 1 mL/min, the oven at 50°C for 30 s, ramp at 4°C/min to 70°C, and ramp at 25°C/min to 240°C.

Quantitative Real-Time PCR (qRT-PCR)

The total RNA from *E. coli* cells grown for 24 h in shake flasks was isolated using an RNA extraction kit (Dongsheng Biotech, Guangzhou, China), following the manufacturer's instructions. The first-strand cDNA was synthesized using an All-in-One™ First-Strand cDNA Synthesis kit (GeneCopoeia, Guangzhou, China). The qRT-PCR was perfor1med with the All-in-One™ qPCR Mix kit (GeneCopoeia) on an iCycler iQ5 Real Time PCR system (Bio-Rad Laboratories, California, USA). The template was 100 ng of cDNA. The PCR conditions were as follows: 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 15 s. The primers for qRT-PCR are presented in Supplementary Table 1. The data were analyzed by the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001) and normalized by *cysG* gene expression.

Gene copy numbers were measured by qPCR on genomic DNA isolated from the appropriate CIChE strains. qPCR was performed as described above. The primers QPt1F/QPt1R and QHF/QHR (Supplementary Table 1) were used to measure the copy number of *Pt1* and *HMGS*, respectively.

Statistical Analysis

All experiments were conducted in triplicate, and the data were averaged and presented as the means ± standard deviation. One-way analysis of variance followed by Tukey's test was used to determine significant differences using the OriginPro (version 7.5) package. Statistical significance was defined as $p < 0.05$.

RESULTS

Tolerance Engineering to Improve Pinene Production

To improve pinene tolerance, *E. coli* cells harboring pP_{rstA}-GFP were treated with ARTP and then serially transferred into LB medium supplemented with increased concentrations of pinene of 0.5, 1.0, 1.5, and 2.0%. The culture was transferred daily. After the adaptive evolution at 2.0% pinene, the culture was streaked on LB plates for isolated colonies. It has been demonstrated that the IPP/FPP sensor plasmid pP_{rstA}-GFP has been successfully used to test the intracellular IPP/FPP concentration and to screen the library with higher IPP/FPP concentrations (Dahl et al., 2013; Shen et al., 2016). Thus, we also used it to screen the library. Of the 670 clones, 14 strains with higher fluorescence strength (Supplementary Figure 1) were selected for further shake flask analysis. As shown in **Figure 1**, *E. coli* YZ-3 produced the highest level of pinene (7.3 ± 0.2 mg/L), which was 31% higher than the starting strain *E. coli* BW25113 (P_{T5}-dxs).

To improve pinene production, we investigated effects of efflux pumps on pinene production. Dunlop et al. (2011) reported that expressing some efflux pumps significantly improved pinene tolerance. Thus, we tested whether pumps that improved pinene tolerance also enhanced its production. As shown in **Figure 2A**, expressing native AcrB, AcrAB, or TtgB (NP_743544) from *Pseudomonas putida* KT2440 in *E. coli* YZ-3 using plasmid

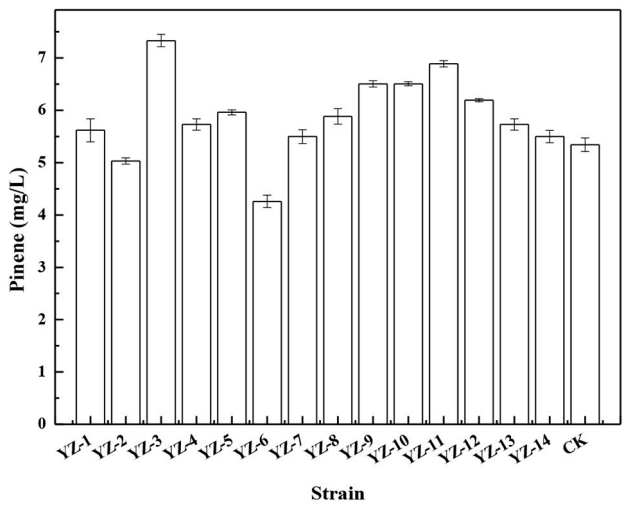

FIGURE 1 | Pinene production by the selected adaptive laboratory evolution strains harboring pMEVIGPS. *E. coli* BW25113 (P_{T5}-dxs, pMEVIGPS) was set as the control strain (CK). The data represent the means of three replicates and error bars represent standard deviations.

resulted in increased pinene production. However, expressing *A. borkumensis* AcrBDFa (YP_692684) or *P. putida* KT2440 MexF (NP_745564) from did not improve pinene production. Therefore, we first replaced the native promoter of *E. coli* YZ-3 *acrAB* operon with the strong P37 promoter to obtain *E. coli* YZ-3-A, resulting in an increase in pinene production to 8.1 ± 0.2 mg/L from 7.3 ± 0.2 mg/L (**Figure 2B**). Then, we integrated the *ttgB* from *P. putida* KT2440 under the control of the P37 promoter in *E. coli* YZ-3-A to obtain *E. coli* YZ-3-A-T. The modification further improved pinene production to 9.1 ± 0.2 mg/L (**Figure 2B**). These results indicate that overexpressing some efflux pumps (*E. coli acrAB* and *Pseudomonas putida* KT2440 *ttgB*), whcih improved pinene tolerance, also enhanced its production.

To further improve pinene production, we isolated a mutant resistant to an inhibitor of biosynthetic pathway after ARTP mutagenesis. Isolating a mutant resistant to an inhibitor of biosysnthetic pathway is a common strategy used for strain improvement. In *E. coli,* the important precursors IPP and DMAPP are produced by the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway. Fosmidomycin is the DXP pathway inhibitor that inhibits 1-deoxy-D-xylulose-5-phosphate reductoisomerase (Dxr) and methylerythritol phosphate cytidyltransferase (IspD) of the DXP pathway (Zhang et al., 2011). Genes involved in the DXP pathway are essential for *E. coli* growth. The wild-type *E. coli* YZ-3-A-T can grow in the presence of 2% pinene (Supplementary Figure 2A), but does not grow in the presence of 35 μM fosmidomycin (Supplementary Figure 2B). After ARTP mutagenesis, cells grow well in the presence of 35 μM fosmidomycin (Supplementary Figure 2C). Overexpression of *dxr* or *ispD* in *E. coli* improved the fosmidomycin tolerance (Zhang et al., 2011). This indicates that the fosmidomycin resistant mutants may show higher level of Dxr and IspD. Screening the fosmidomycin resistant mutants will increase the probability to obtain a mutant with higher IPP flux. Thus, to increase the probability to obtain a mutant with higher IPP flux, we screened the fosmidomycin resistant mutants using the IPP/FPP sensor. *E. coli* YZ-3-A-T cells harboring pP_{rstA}-GFP were treated with ARTP. After ARTP mutagenesis, the cells were transferred into the LB medium supplemented with 35 μM fosmidomycin and 2.0% pinene. A total of 720 clones were screened for analyzing fluorescence strength in deep-well microplate cultures (Supplementary Figure 3). Twenty-one strains with higher fluorescence strength were selected for further shake flask analysis. As shown in **Figure 3**, Strain No. 19, which was denoted as *E. coli* YZFP, produced the highest level of pinene, which reached 9.9 ± 0.1 mg/L. In fact, our quantitative real-time PCR analysis also demonstrates that the *dxs*, *dxr* and *ispD* of the DXP pathway in *E. coli* YZFP showed higher transcription level than the wild-type strain (Data not shown, will be published in another paper).

To characterize the pinene tolerance, the growth of the above strains were compared in different concentrations of pinene. **Figure 4A** shows the growths of these strains in the presence of 2% pinene. The starting strain did not grow well in the presence of 2% pinene. The above engineered strains did grow well in the presence of 2% pinene. The maximum cell densities of the

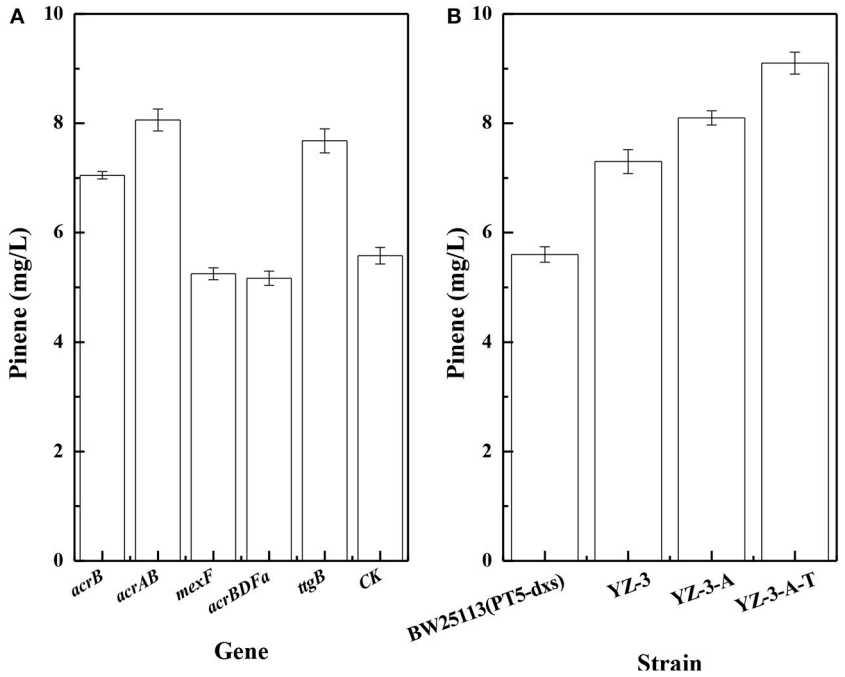

FIGURE 2 | Effect of overexpression of efflux pumps on pinene production. **(A)** Plasmid-expression in *E. coli* YZ-3 (pMEVIGPS). *E. coli* YZ-3 (pMEVIGPS, pZEABP) was set as the control strain (CK); **(B)** Chromosomal-expression in *E. coli* harboring pMEVIGPS. The data represent the means of three replicates and error bars represent standard deviations.

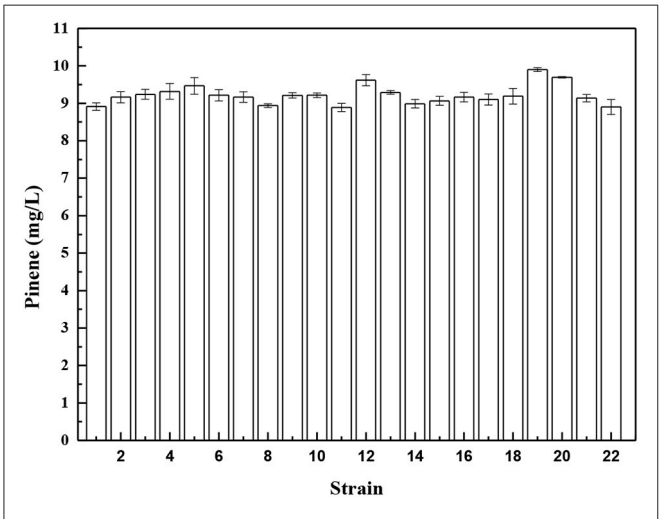

FIGURE 3 | Pinene production of the selected mutants resistant to fosmidomycin harboring pMEVIGPS. *E. coli* BW25113 (P_{T5}-dxs, pMEVIGPS) (strain No. 22) was set as the control strain. The data represent the means of three replicates and error bars represent standard deviations.

three engineered strains were similar. The growth rate of *E. coli* YZFP was higher than that of the other engineered strains. These results indicate that the engineered strains have higher pinene tolerance than the starting strain. However, the maximum cell densities of the three engineered strains were lower than that of the starting strain in the absence of pinene (**Figure 4B**). The reason may be that the three engineered strains produced higher level of IPP than the starting strain. IPP is toxicity to *E. coli*. We also investigated the genetic stability of *E. coli* YZFP. The strain can also grow well in the presence of 2% pinene and the level of pinene production remained constant after 20 rounds of subculturing in absence of selective pressure (data not shown).

Evolution Engineering to Improve Pinene Production

The lower expression level and/or lower enzymatic activity of GPPS and PS in *E. coli* may result in the lower yield of pinene production. Sarria et al. (2014) compared GPPSs and PSs from *A. grandis* and *P. taeda* and found that the combination of GPPS and PS from *A. grandis* was most suitable for pinene production. Thus, we first optimized the first 48 nucleotide sequences of *A. grandis GPPS* with the 6AA method to increase the expression level of the *A. grandis GPPS-PS* gene cluster in *E. coli*. The 6AA method substitutes all Arg, Asp, Gln, Glu, His, and Ile codons with the synonymous codon having the highest single-variable logistic regression slope (CGT, GAT, CAA, GAA, CAT, and ATT, respectively), while the other 14 amino acids were not changed from the wild-type gene sequence (Boë et al., 2016). The 6AA optimization increased pinene production from 5.6 ± 0.1 mg/L to 6.4 ± 0.3 mg/L (**Table 2**).

Because pinene shares the same 5-carbon precursors IPP and DMAPP with carotenoids, a lycopene-producing strain *E. coli* LYCOP (Chen et al., 2013) was used to screen the error-prone

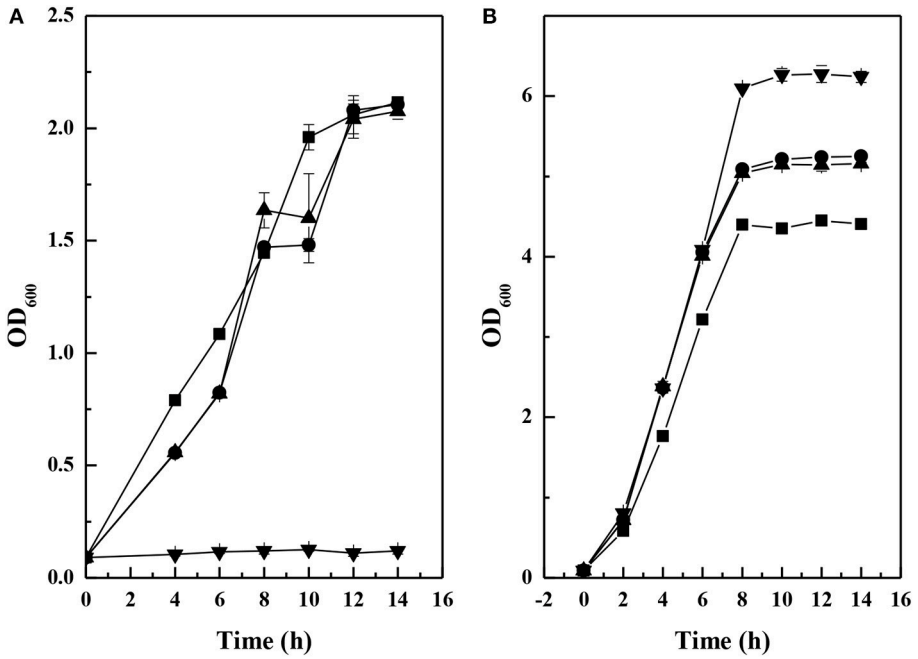

FIGURE 4 | Growth of the selected tolerance strains in the presence of 2% pinene **(A)** and in the absence of pinene **(B)**. *E. coli* BW25113 (P_{T5}-dxs) (▼), *E. coli* YZ-3 (▲), *E. coli* YZ-3-A-T (●), and *E. coli* YZFP (■). The data represent the means of three replicates and error bars represent standard deviations.

TABLE 2 | Effect of evolution engineering on pinene production in *Escherichia coli* BW25113 (P_{T5}-dxs, pMEVI).

Gene cluster	Genetic modification	OD_{600}	Pinene concentration (mg/L)
AgGPPS-AgPS	Wild-type	12.30 ± 0.43	5.6 ± 0.1 (100.0%)
$AgGPPS_{6AA}$-*AgPS*	The first 18 codons of *A. grandis* GPPS were optimized by using the 6AA method	12.22 ± 0.41	6.4 ± 0.3 (114.3%)
$AgGPPS_{6AA}$-$AgPS^{epPCR}$	The fusion *GPPS-PS* gene cluster variant from *A. grandis* after error-prone PCR	12.23 ± 0.39	10.4 ± 0.3 (185.7%)
$AgGPPS^{MUT}$-$AgPS^{DNA\ shuffling}$	The fusion *GPPS-PS* gene cluster variant from *A. grandis* after DNA shuffling	12.21 ± 0.45	12.4 ± 0.2 (221.4%)
$AgGPPS^{mut}$-$Pt1^{Q457L}$	The fusion gene cluster of the $GPPS^{D90G/L175P}$ and $Pt1^{Q457L}$	12.10 ± 0.38	15.2 ± 0.2 (271.4%)
$AgGPPS^{MUT}$-TIGR-$Pt1^{Q457L}$	The TIGR-mediated gene cluster of the GPPS and $Pt1^{Q457L}$	12.11 ± 0.37	17.6 ± 0.2 (314.3%)

Data represent the means of three replicates and standard deviations.

PCR mutant libraries of the GPPS-PS cluster from *A. grandis* after 6AA optimization. The higher the activity of the GPPS-PS cluster, the lower the intracellular precursor levels for lycopene biosynthesis, thereby reducing the pigmentation of the *E. coli*. Of approximately 1 500 colonies, 7 colonies with a whiter color were observed. The mutant plasmids were isolated from the 7 colonies and then were co-transferred with the MEV pathway plasmid pMEVI into *E. coli* BW25113 (P_{T5}-dxs). The pinene productions of them were analyzed in a shake flask, and the results are presented in **Figure 5A**. The strains harboring the mutant gene cluster produced higher pinene by 7.8–85.7% than that with the wild-type gene cluster. To increase the gene cluster activity, the 7 mutant gene clusters were used for DNA shuffling.

Because the colonies of *E. coli* LYCOP harboring the above mutant gene cluster became a faint color, it is difficult to discriminate these colonies by using the above carotenoid-based method. A more sensitive and quantitative screening method is needed. It is known that monoterpene can hydrate readily in the presence of acid catalysts, such as H_2SO_4 (Robles-Dutenhefner et al., 2001). As a result, the initial reaction solutions turn yellow and then brown. After reaction with concentrated sulfuric acid in boiling water for 5 min, it was observed that the color of the reaction solution become darker as the pinene concentration increases and the absorbance at 450 nm is linearly related with pinene concentration (Supplementary Figure 4). Thus, the concentrated sulfuric acid method can quantitatively predict pinene concentrations.

After DNA shuffling, the mutant plasmids were transferred into *E. coli* LYCOP harboring pMEVI. Fifty colonies with a whiter color were used for assays of pinene production in a shake flask using the concentrated sulfuric acid method. The results are presented in **Figure 5B**. *E. coli* LYCOP harboring the mutant gene cluster produced higher pinene (6.5–10.1 mg/L) than those

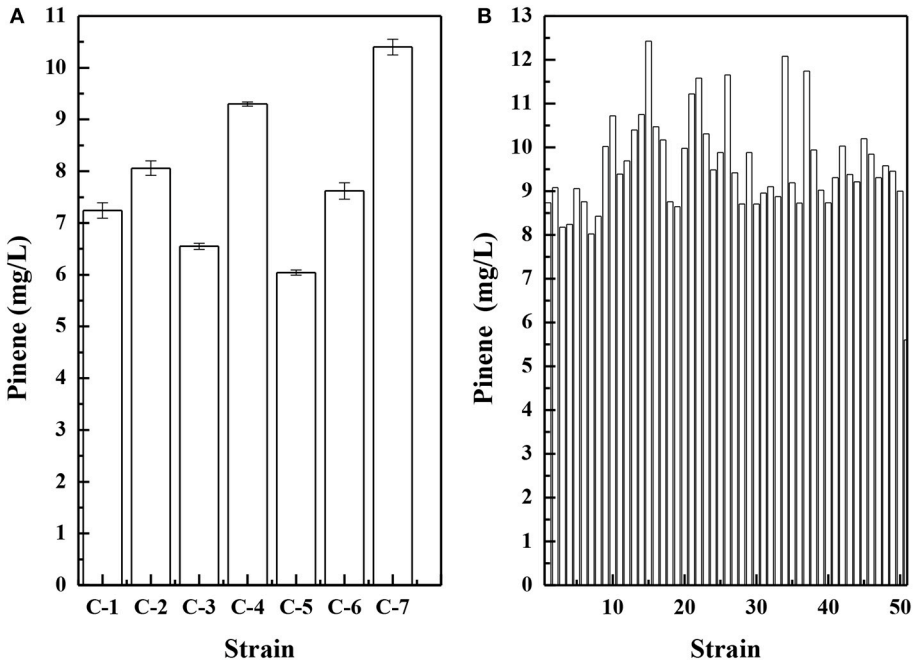

FIGURE 5 | Pinene production by *E. coli* BW25113 (P_{T5}-dxs, pMEVI) harboring mutant gene clusters from error-prone PCR **(A)** and by *E. coli* LYCOP harboring mutant gene clusters from DNA shuffling **(B)**. Pinene concentrations were measured using the GC-FID **(A)** and the concentrated sulfuric acid **(B)** methods. The data represent the means of three replicates and error bars represent standard deviations.

with the wild-type gene cluster. The mutant plasmid with the highest pinene production was isolated from strain No. 15 and then was co-transferred with pMEVI into *E. coli* BW25113 (P_{T5}-dxs). *E. coli* BW25113 (P_{T5}-dxs) harboring the mutant plasmid and pMEVI produced 12.4 ± 0.2 mg/L of pinene (**Table 2**). The mutant plasmid with the highest pinene production was then sequenced. The two amino acid mutants (D90G and L175P) were observed in the CDS of *GPPS* from *A. grandis*. No mutant in the CDS of *PS* from *A. grandis* was observed. It has been reported that (-)-α-pinene synthase (Pt1) from *P. taeda* has the lowest K_m for GPP among the known PSs (Phillips et al., 2003). Tashiro et al. engineered an *E. coli* with the highest yield of pinene so far using the pinene synthase mutant ($Pt1^{Q457L}$) from *P. taeda* (Tashiro et al., 2016). Thus, replacing *A. grandis PS* in the mutant plasmid with *P. taeda* pinene synthase mutant gene ($Pt1^{Q457L}$) yielded pQE30-$GPPS^{mut}$-L-$Pt1^{Q457L}$. *E. coli* BW25113 (P_{T5}-dxs) harboring pQE30-$GPPS^{mut}$-L-$Pt1^{Q457L}$ produced a higher level of pinene (15.2 ± 0.2 mg/L) than those harboring pQE30-$GPPS^{mut}$-L-AgPS, which achieved 12.4 ± 0.2 mg/L (**Table 2**).

The unbalanced expression of multiple genes may overburden the cell and cause accumulation of toxic metabolic intermediates, resulting in reduced product titers. Pfleger et al. (2006) developed a combinatorial engineering approach for coordinating the expression of cascade enzymes. For this purpose, libraries of tunable intergenic regions (TIGRs) are generated that encode mRNAs with diverse secondary structures with RNase cleavage sites. The TIGR approach was applied to balance the gene expression of the MEV pathway using the TIGR approach, resulting in a 7-fold increase in mevalonate production. Moreover, our previous paper demonstrated that the TIGR approach was more efficient compared to protein fusion for coordinating expression (Li et al., 2015). Thus, we constructed a library of TIGRs to balance the expression of *A. grandis* $GPPS^{D90G/L175P}$ and *P. taeda* $Pt1^{Q457L}$. The library of TIGRS was inserted between $GPPS^{D90G/L175P}$ and *P. taeda* $Pt1^{Q457L}$ to yield a series of operons. The functional operons from the libraries were screened by using the concentrated sulfuric acid method. A total of 768 colonies were used for the assay of pinene production in deep-well microplate cultures using the concentrated sulfuric acid method (Supplementary Figure 5). Forty-three strains with higher OD_{450} were selected for further shake flask analysis. As shown in **Figure 6**, strain No. 6 produced the highest level of pinene (17.6 ± 0.2 mg/L). Thus, the TIGR-mediated plasmid was recovered from strain No. 6 and sequenced (Supplementary Table 2). We also retransformed the plasmid back to the host strain *E. coli* BW25113 (P_{T5}-dxs) and checked the pinene production. The resulting strain produced the same level of pinene (17.9 ± 0.1 mg/L), indicating that the pinene production improvement is the result of TIGR-mediated optimization.

Modular Co-culture Engineering to Improve Pinene Production

To take advantage of emerging co-culture engineering approaches to improve overall pinene biosynthesis in *E. coli*, the complete biosynthetic pathway was divided into the following two modules: the upstream module of the MEV pathway and the downstream module of the TIGR-mediated gene cluster of *A. grandis* $GPPS^{Mut}$ and *P. taeda* $Pt1^{MUT}$ (**Figure 7**). The two

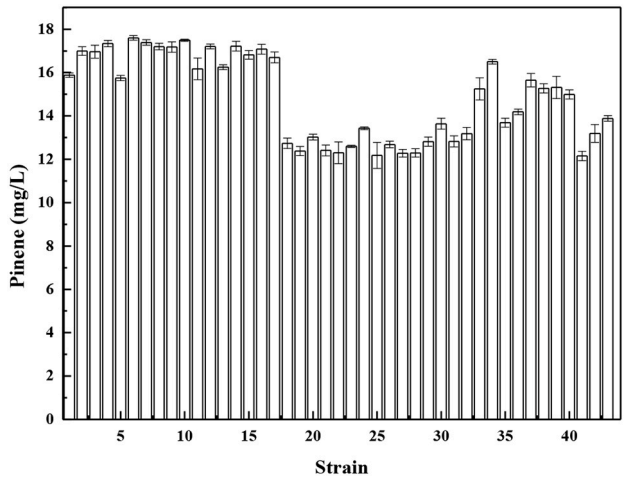

FIGURE 6 | Pinene production by *E. coli* BW25113 (P_{T5}-dxs, pMEVI) harboring the selected TIGR-mediated gene cluster. The data represent the means of three replicates and error bars represent standard deviations.

modules were integrated into the chromosome of the pinene tolerance strain *E. coli* YZFP and then then evolved to a higher gene copy number by triclosan induction, respectively.

Figure 8A shows the results of pinene production in CIChE strains of the TIGR-mediated gene cluster of *A. grandis GPPS*^{Mut} and *P. taeda Pt1*^{MUT} without the MEV pathway. The maximum pinene production was obtained by the CIChE strains resistant to 32 μM triclosan. Thus, the *recA* gene of the CIChE strain resistant to 32 μM triclosan was deleted to obtain *E. coli* PINE. We determined the *GPPS-Pt1* gene copy number in *E. coli* PINE. The copy number reached approximately 60 in the CIChE strain, which is the equivalent copy number of a high copy plasmid. **Figure 8B** shows the results of IPP/FPP concentration of the CIChE strains of the MEV pathway measured by the IPP/FPP sensor (pP_{rstA}-GFP). As shown in **Figure 8B**, the maximum IPP/FPP production was obtained by the CIChE strains resistant to 0.5 μM triclosan. Thus, the *recA* gene of the CIChE strain resistant to 0.5 μM triclosan was deleted to obtain *E. coli* MEVI. We also determined the MEV pathway gene copy number in *E. coli* MEVI. The copy number reached approximately 4 in *E. coli* MEVI.

Zhou et al. (2015) demonstrate that the modular co-culture engineering can be applicable to isoprenoids because their scaffold moleculars can generally permeate membranes. To demonstrate IPP can also cross cell membranes, we cultured *E. coli* (pP_{rstA}-GFP) with the cell-free culture broth of *E. coli* MEVI and measured fluorescence strength. After addition of the cell-free culture broth of *E. coli* MEVI, *E. coli* (pP_{rstA}-GFP) showed higher fluorescence strength (Supplementary Figure 6). Moreover, the *E. coli* MEVI: PINE co-culture produced higher level of pinene than *E. coli* PINE (**Figure 9**). These results indicate that IPP produced by *E. coli* MEVI diffused into *E. coli* PINE and was subsequently converted into pinene. We then optimized the *E. coli* MEVI: PINE co-culture system to further improve pinene production. To this end, different inoculation ratios between *E. coli* MEVI and PINE were investigated. As shown in **Figure 9**, the highest pinene production of 64.9 ± 0.9 mg/L was achieved when *E. coli* MEVI and PINE were inoculated at a ratio of 1:2. Compared with the mono-culture strategy using *E. coli* PINE harboring pMEVI, the pinene production was increased by 1.9-fold (from 22.3 ± 0.2 mg/L to 64.9 ± 0.9 mg/L). To test if all of IPP produced by *E. coli* MEVI were converted into pinene by *E. coli* PINE, we measured the IPP concentration in the broth of the co-culture and the *E. coli* MEVI after 28 h using the IPP sensor plasmid. The results showed that about 57.8% of IPP were converted by *E. coli* PINE (Supplementary Table 3). Thus, we introduced pQE-GPPS^{MUT}-TIGR-Pt1^{Q457L} to overexpress the pinene biosynthetic pathway and checked the pinene production. The co-culture system after introducing the pinene biosynthetic pathway into *E. coli* MEVI produced higher level of pinene (60.2 ± 0.2 mg/L) than the control strain (52.1 ± 0.1 mg/L) harboring the empty plasmid (Supplementary Table 4). The result also demonstrates that not all of IPP can be converted in the co-culture system.

Biotechnological approaches for chemicals production can be broadly classified into fermentation and biocatalysis. In biocatalysis, cell growth and production phase are separated. In comparison to the fermentation bioprocess, whole-cell biocatalysis is an attractive method due to its great efficiency and relative simplification of downstream processing (Lin and Tao, 2017). The whole-cell biocatalysis processes comprise the following two stages: growth and conversion of the substrates. After the cells are cultured, they are harvested and washes with a buffer solution and suspended in the buffer for biocatalysis. Thus, the *E. coli-E. coli* modular co-culture system of whole-cell biocatalysis was used to further enhance pinene production. As shown in **Figure 10**, the highest pinene production of 166.5 ± 0.3 mg/L was achieved by the whole-cell biocatalyst after 28 h. The pinene titer obtained by the whole-cell biocatalysis was 2.6-fold higher than that produced by the fermentation process.

DISCUSSION

It has been reported that *E. coli* growth is inhibited by 0.5% pinene (Dunlop et al., 2011). We first improved pinene tolerance from 0.5 to 2.0% and pinene production by adaptive laboratory evolution after ARTP mutagenesis. In fact, improvements in tolerance are not sufficient to guarantee an increase production. Our results also demonstrate this point. Overexpreesion of *A. borkumensis acrBDFa* or *P. putida* KT2440 *mexF* that improved pinene tolerance did not improved pinene production (**Figure 2A**). To obtain a mutant with higher level of pinene production, we used the IPP/FPP sensor pP_{rstA}-GFP to screen the mutants tolerant to 2% pinene. Tolerance engineering has also successfully been used to improve the production of limonene (Dunlop et al., 2011), amorphadiene (Zhang et al., 2016), olefin (Mingardon et al., 2015), n-octane (Foo and Leong 2013). Although the level of pinene production reported in literatures did not inhibit growth, higher tolerance is beneficial to pinene production. Thus, the 2% pinene tolerant strain *E. coli* was used the parent strain in this study. To further improve pinene

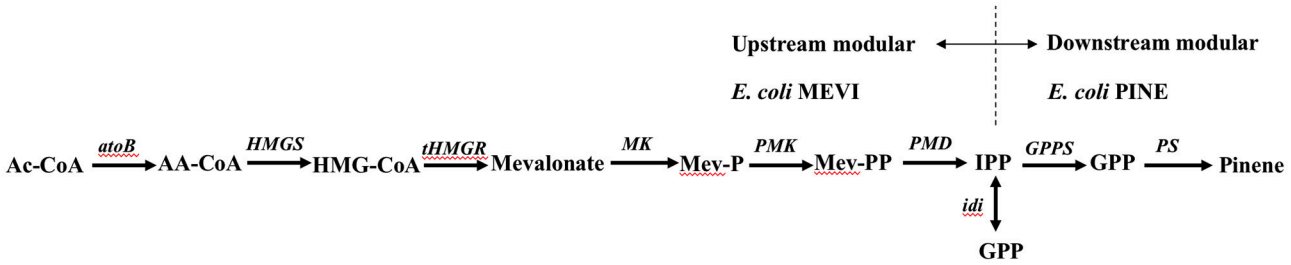

FIGURE 7 | Strategy of modular co-culture engineering.

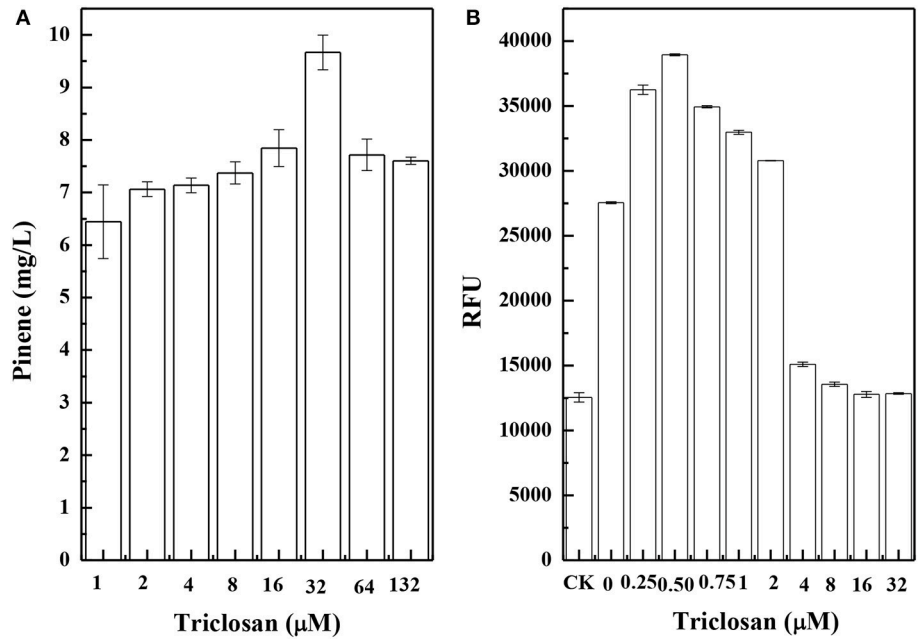

FIGURE 8 | Pinene production of chemically induced chromosomal evolution (ClChE) strains of the GPPS-Pt1 cluster without the MEV pathway **(A)** and the MEV pathway **(B)** at different triclosan concentrations. The data represent the means of three replicates and error bars represent standard deviations.

production, we then expressed the efflux pumps in the pinene tolerant strain *E. coli* and subsequently selected a mutant resistant to fosmidomycin after ARTP mutagenesis. The pinene tolerant strain *E. coli* YZFP with higher level of pinene production was obtained through a two-step screening process. There is no directed evidence to prove the improved pinene production is the result of improved pinene tolerance.

Our study demonstrates that the overexpression of some efflux pumps improved pinene tolerance and production. Many groups also reported that overexpression of efflux pumps enhanced biofuel tolerance. Dunlop et al. (2011) reported that the overexpression of efflux pumps, such as *A. borkumensis* AcrBDFa, *P. putida* KT2440 MexF, *P. putida* KT2440 TtgB or *E. coli* AcrB, enhanced pinene tolerance. However, they did not investigate the effects of the pumps on pinene production. Our results demonstrate that overexpression of *E. coli* AcrAB and *P. putida* KT2440 TtgB enhanced pinene production (**Figure 2**). Overexpression of *A. borkumensis* AcrBDFa or *P. putida* KT2440 MexF did not improved pinene production (**Figure 2A**). However, Dunlop et al. (2011) reported that overexpression of *A. borkumensis* AcrBDFa enhanced limonene tolerance and yield. Overexpression of *tolC* together with ABC family transporters (*macAB*) or MFS family transporters (*emrAB* or *emrKY*) was found to improve amorphadiene titer by more than 3-fold (Zhang et al., 2016). Overexpression of the native and evolved *acrB* improved olefin tolerance and production (Mingardon et al., 2015). Evolved AcrB variants with improved tolerance to pinene and n-octane have also been reported by Foo and Leong (2013). Taken together with these previous studies, our results show that a combination of the adaptive laboratory evolution with overexpression of some efflux pumps can improve pinene tolerance and production.

In this study, we reported a high-throughput screening method, which is known as the concentrated sulfuric acid method, for recombinant *E. coli* that overproduce pinene. We successfully applied the concentrated sulfuric acid method to

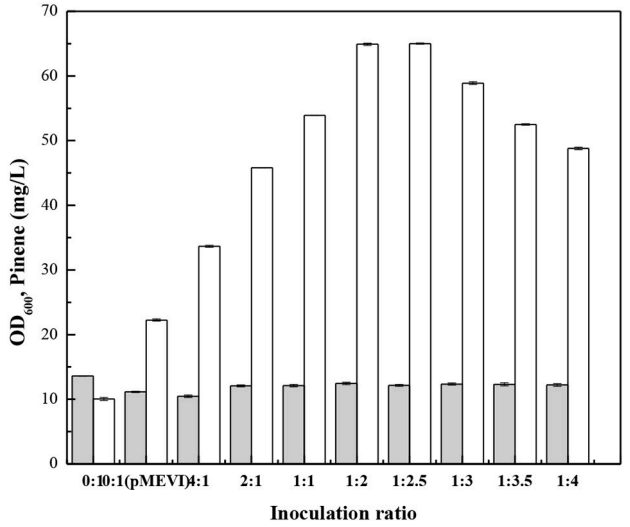

FIGURE 9 | Effect of the inoculation ratio of *E. coli* PINE and MEVI on pinene production in the co-culture system. OD_{600} (Gray bars), Pinene concentration (White bars). 0:1, only *E. coli* PINE; 0:1 (pMEVI), only *E. coli* PINE (pMEVI); others, the *E. coli* MEVI: PINE co-culture system with different inoculation ratio. The data represent the means of three replicates and error bars represent standard deviations.

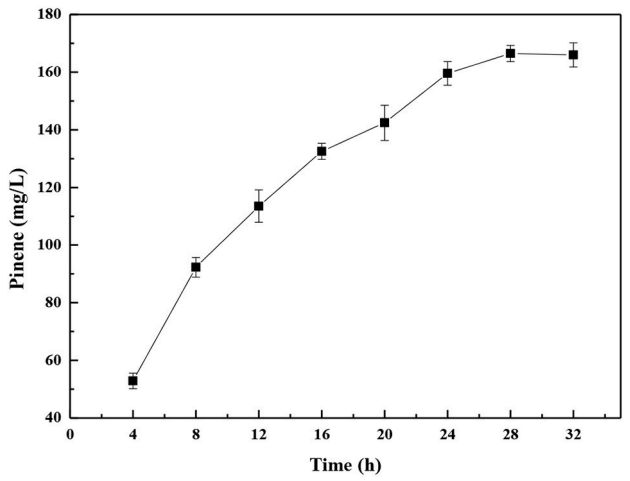

FIGURE 10 | Time course of pinene production by the modular co-culture system of the whole-cell biocatalyst. The data represent the means of three replicates and error bars represent standard deviations.

screen the DNA shuffling library of the GPPS-PS gene cluster and the library of the TIGR-mediated *GPPS-Pt1* gene cluster. Because limonene has the same properties as pinene, the concentrated sulfuric acid method can also be used to screen mutants for limonene production. Although the carotenoid-based method has been successfully used to screen isoprene synthase variants (Emmerstorfer-Augustin et al., 2016), the carotenoid-based method has a limitation when the colony has a faint color.

GPPS and PS have been identified as a major limiting factor in pinene production (Yang et al., 2013; Sarria et al., 2014; Tashiro et al., 2016). After directed evolution of the *A. grandis GPPS-PS* gene cluster using error-prone PCR and DNA shuffling, pinene production was increased by 1.2-fold (**Table 2**). Two amino acid mutants were observed in the CDS of *A. grandis GPPS*. However, no mutant was observed in the CDS of *A. grandis PS*. Tashiro et al. evolved *P. taeda Pt1* and constructed a recombinant *E. coli* with the highest pinene yield reported in literatures using the evolved variant (Tashiro et al., 2016). Using the *A. grandis* $GPPS^{Mut}$-*P. taeda* $Pt1^{MUT}$ gene cluster resulted in an increase in pinene production by 22.6% compared to using the *A. grandis* $GPPS^{Mut}$-PS gene cluster (**Table 2**).

GPPS and PS are inhibited by their substrate (GPP) or product (pinene) (Sarria et al., 2014). To overcome GPPS inhibition by GPP, GPPS was fused to PS, resulting in improved pinene production (Sarria et al., 2014; Tashiro et al., 2016). Our previous paper demonstrated that the TIGR approach was more efficient compared to protein fusion for coordinating expression (Li et al., 2015). This study shows that using the TIGR-mediated gene cluster led to an increase in pinene production by 15.8% compared with the fused gene cluster (**Table 2**).

In the present study, an *E. coli-E. coli* co-culture system was engineered to modularize the MEV and heterologous biosynthetic pathway. The MEV pathway and heterologous biosynthetic pathway (the *A. grandis* $GPPS^{Mut}$-*P. taeda* $Pt1^{MUT}$ gene cluster) was engineered in the pinene tolerance strain *E. coli* YZFP, respectively. The best co-culture system was found to improve pinene production by 1.9-fold compared to the mono-culture system. The modular co-culture can distribute the metabolic burden and allow for modular optimization by simply changing the strain-to-strain ratio. The *E. coli-E. coli* modular co-culture system has been successfully used to improve 3-amino-benzoic acid (Zhang and Stephanopoulos, 2016), flavonoid (Jones et al., 2016), muconic acid (Zhang et al., 2015), and perillyl acetate (Willrodt et al., 2015), etc. In fact, the critical issue for modular co-culture engineering is the mass transfer of the pathway intermediate (IPP). It has been demonstrated that isoprenoids scaffold molecules can cross cell membranes (Zhou et al., 2015). Our results also demonstrate that IPP can cross cell membranes and secreted to the extracellular medium (Supplementary Figure 6 and **Figure 9**). Moreover, our results showed that the pinene tolerance strain *E. coli* YZFP (pP_{rstA}-GFP) had higher fluorescence strength than the parent strain harboring pP_{rstA}-GFP after addition the cell-free broth of *E. coli* MEVI (Supplementary Figure 6), indicating that *E. coli* YZFP shows greater membrane permeability than the parent strain. Our results demonstrate that there were still some IPP not to be converted into pinene by *E. coli* PINE (Supplementary Tables 3, 4). Moreover, Overexpression of the pinene biosynthetic pathway in *E. coli* MEVI enhanced pinene production in the *E. coli* MEVI-*E. coli* PINE co-culture system (Supplementary Table 4). However, overexpression of the pinene biosynthetic pathway in *E. coli* PINE did not enhance pinene production (Supplementary Table 4). Increasing the inoculation ratio of *E. coli* PINE and *E. coli* MEVI from 2:1 to 2.5:1 or 3:1 did not enhanced pinene production (**Figure 9**). These results

indicate that the IPP transportation may be a key factor for further improving pinene production. Transporter engineering strategies have successfully been used to enhance the secretion of the pathway intermediates, improving production (Boyarskiy and Tullman-Ercek, 2015; Kell et al., 2015; Zhang et al., 2015). Thus, appropriate metabolite transporters engineering strategies may be used to further improve pinene production of the *E. coli-E. coli* co-culture system.

This study also demonstrated that whole-cell biocatalysis further improved pinene production by 1.6-fold compared to the fermentation process. The whole-cell biocatalysis has also successfully been used in many biotechnological production (Tao et al., 2011; Lin et al., 2015; Kogure et al., 2016; Chen et al., 2017; Lin and Tao, 2017). Kogure et al. (2016) also reported that the significantly higher shikimate productivity (141.3 g/L) was achieved by the whole-cell biocatalysis compared to that (78.8 g/L) achieved by the fed-batch fermentation accompanying cell growth. The pinene production improvement may be resulted from higher cell density (OD_{600} of 30) and the growth-arrested cells used in the whole-cell biocatalysis.

CONCLUSIONS

Pinene tolerance and production were first improved via adaptive laboratory evolution and efflux pump overexpression. Through error-prone PCR and DNA shuffling, a GPPS variant was screened, which outperformed the wild-type enzyme. To balance the expression of multiple genes, a TIGR was inserted between *A. grandis* $GPPS^{D90G/L175P}$ and *P. taeda* $Pt1^{Q457L}$. To construct an *E. coli-E. coli* co-culture system to modularize the MEV and heterologous biosynthetic pathway, the MEV pathway and heterologous biosynthetic pathway (the *A. grandis* $GPPS^{Mut}$-*P. taeda* $Pt1^{MUT}$ gene cluster) was integrated into the chromosome of the pinene tolerance strain *E. coli* YZFP and then evolved to a higher gene copy number by CIChE, respectively. The *E. coli-E. coli* modular co-culture system of whole-cell biocatalysis resulted in the highest pinene production of 166.5 mg/L. Our results demonstrate that the *E. coli-E. coli* modular co-culture system of the whole-cell biocatalysis is a promising approach for the production of pinene.

AUTHOR CONTRIBUTIONS

F-XN performed all of the experimental works. XH and Y-QW performed the pinene assay. J-ZL designed the study and wrote the manuscript. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01623/full#supplementary-material

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REVIEW
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Heterologous Production of Microbial Ribosomally Synthesized and Post-translationally Modified Peptides

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Ribosomally synthesized and post-translationally modified peptides, or RiPPs, which have mainly isolated from microbes as well as plants and animals, are an ever-expanding group of peptidic natural products with diverse chemical structures and biological activities. They have emerged as a major category of secondary metabolites partly due to a myriad of microbial genome sequencing endeavors and the availability of genome mining software in the past two decades. Heterologous expression of RiPP gene clusters mined from microbial genomes, which are often silent in native producers, in surrogate hosts such as *Escherichia coli* and *Streptomyces* strains can be an effective way to elucidate encoded peptides and produce novel derivatives. Emerging strategies have been developed to facilitate the success of the heterologous expression by targeting multiple synthetic biology levels, including individual proteins, pathways, metabolic flux and hosts. This review describes recent advances in heterologous production of RiPPs, mainly from microbes, with a focus on *E. coli* and *Streptomyces* strains as the surrogate hosts.

Keywords: RiPPs, heterologous expression, precursor peptide, processing enzymes, synthetic biology, *E. coli*, *Streptomyces*

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INTRODUCTION

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a large group of natural products with a high degree of structural diversity and a wide variety of bioactivities (**Figure 1A**; Arnison et al., 2013). So far, over 20 different families of RiPPs have been discovered, each carrying unique chemical features (Ortega and van Der Donk, 2016). A biosynthetic logic for RiPPs has emerged and can be simplified as the post-translational modification (PTM) of ribosomally synthesized precursor peptides (**Figure 1B**; Arnison et al., 2013). An ever-growing list of PTMs expand chemical functionality and often impart metabolic and chemical stability upon precursor peptides. One precursor peptide usually contains the leader peptide (in rare cases *C*-terminal, named as follower peptide) *N*-terminal to the core peptide. The leader peptide binds to and guides biosynthetic enzymes for PTMs on the core peptide and is eventually removed from the modified core peptides by proteases. The entire sequence of the core peptide is generally retained in the final structures of RiPPs and can carry multiple variable sites. As such, the separation of substrate recognition and catalysis enables a concise RiPP biosynthetic route, possessing an evolutionary advantage of accessing high chemical diversity at low genetic cost.

As a consequence of their ribosomal origin, the chemical structures of RiPPs are more predictable from genomic data than other families of natural products, making RiPPs an attractive target of genome-driven natural product discovery efforts. Compared to conventional "top-down" approaches, the starting point of the genome-driven approach is genome sequences that have exponentially grown over the past decade. Many specialized bioinformatic tools have been developed for identifying RiPPs biosynthetic gene clusters, such as AntiSMASH (Weber et al., 2015), PRISM (Skinnider et al., 2017), SMURF (Khaldi et al., 2010), and more recently RODEO (Tietz et al., 2017). However, there are many technical challenges to translate the identified clusters into chemical entities, rendering the genome-driven approach far from being a panacea for accessing the chemical space that natural products occupy (Luo et al., 2014). Indeed, the diversity and complexity of PTMs, which are often essential for bioactivity of RiPPs, are not readily identifiable on the core peptides as our understanding of biosynthetic enzymes, particularly their substrate specificity and regio-, stereo-, and chemo-selectivity, remains limited (Arnison et al., 2013). On the other hand, the structural determination of RiPPs is often challenged with their no-to-low isolation yields from samples collected from the field or cultured under laboratory conditions (Smith et al., 2018). Over the past decade, many approaches have been developed to address this critical, major issue of the genome-driven approach, including the activation of silent biosynthetic gene clusters (e.g., modification of fermentation methods and engineering of original producers), heterologous expression using a genetically tractable surrogate host, and *in vitro* reconstruction (Chiang et al., 2011; Abdelmohsen et al., 2015; Reen et al., 2015; Ren et al., 2017). Among them, heterologous expression of RiPPs in surrogate hosts, commonly *Escherichia coli* and *Streptomyces* strains, has so far been one of the most successful methods to elucidate cryptic gene clusters and discover new RiPPs (Ortega and van Der Donk, 2016). Furthermore, heterologous production can effectively harvest the promiscuity of RiPP biosynthetic systems to produce designed analogs through genetic engineering of precursor peptides. Importantly, many emerging strategies have been developed to improve the success of heterologous production of RiPPs over the past several years, mainly focusing on the manipulation of individual proteins, pathways, metabolic flux and hosts (**Figure 2**). Herein, this review describes the details of these strategies ensuring and expanding the heterologous expression approach to discover and develop RiPPs. Representative examples of heterologous expression of each major family of RiPPs were summarized in **Table 1**. Of note, thousands of antimicrobial peptides have been isolated from a variety of organisms (Deng et al., 2017), and this manuscript excluded their heterologous production in discussions.

MANIPULATION OF COMPONENTS OF RiPP BIOSYNTHETIC PATHWAYS

A RiPP gene cluster commonly comprises of all essential genes for the production of RiPP. Manipulation of the pathway-specific components allows precise and rational improvement of RiPP production and minimizes potential perturbation of the holistic metabolism of the heterologous host. Detailed information regarding the function, timing, specificity and regulation on the pathway can also be extracted via this approach. From a synthetic biology standpoint, here we use representative examples to describe different strategies used to manipulate RiPP biosynthetic pathways for successful heterologous expression.

Promoter Engineering to Control Gene Transcription

Altered transcription levels of biosynthetic genes are commonly observed when they are introduced into heterologous hosts. Genetic engineering of a biosynthetic gene cluster by the introduction of one or more constitutive or inducible promoters has proved very effective for the heterologous production of different RiPP families. Importantly, a number of well-characterized promoters of commonly used hosts (e.g., *E. coli* and *Streptomyces* strains) (De Mey et al., 2007; Li et al., 2015; Myronovskyi and Luzhetskyy, 2016) have been available to enable this synthetic biology approach. For example, lichenicidin is a two-component lantibiotic produced by *Bacillus licheniformis* I89, and its heterologous production from the native gene cluster in *E. coli* BLic5 led to a significantly lowered yield compared with the native producer (**Table 1**; Caetano et al., 2011a,b). By contrast, driving the expression of each biosynthetic gene by a strong T7 promoter resulted in a yield of lichenicidin up to 100 times higher than *B. licheniformis* I89 (Kuthning et al., 2015). In another example, *Staphylococcus warneri* ISK-1 produces a lantibiotic nukacin ISK-1 (Sashihara et al., 2000) but the heterologous expression of its gene cluster in *S. carnosus* TM300 and *Lactobacillus plantarum* ATCC 14917^{T} failed to produce any natural product (Aso et al., 2004). Aso et al. addressed this problem through the identification of a cognate response activator and by driving the cluster expression with a nisin-inducible promoter PnisA (**Table 1**; Aso et al., 2004). Likewise, the utilization of a proper promoter was also essential for the successful production of a macrocyclic peptide telomestatin (**Table 1**). Initially, a xylose-inducible promoter (xylAp) was used to drive the expression of its gene cluster in the highly engineered *Streptomyces avermitilis* SUKA17 (Komatsu et al., 2013) but yielded no targeted molecule. It was later speculated that the transcription of the gene cluster should be activated during the late logarithmic phase of cell growth. Accordingly, the replacement of xylAp with the olmRp promoter led to the production of telomestatin in *S. avermitilis* SUKA17 (Amagai et al., 2017), clearly indicating the essentiality and importance of temporal control of gene expression in the successful production of natural products. Other remarkable examples of applying constitutive or inducible promoters to promote the success of RiPP heterologous expression include the complete refactoring of the cyanobactin patellamide pathway for its expression in *E. coli* Rosetta2 (DE3) (Donia et al., 2006), the use of inducible araP_{BAD} promoter to drive the entire operon of a lasso peptide in *E. coli* BL21 (DE3) (Metelev et al., 2013), increased production of thiopeptides GE2270 and lactazole A

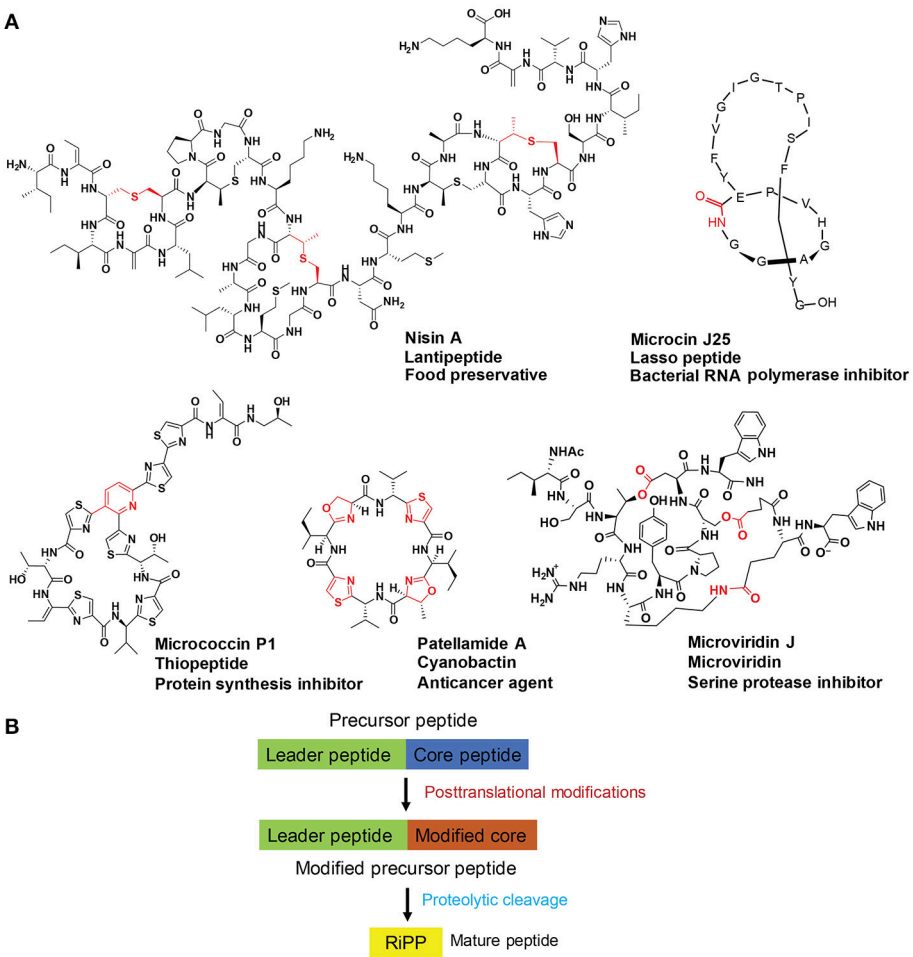

FIGURE 1 | (A) Representative structures of five select RiPP families with diverse bioactivities. Post-translational modification(s) on each structure are highlighted in red. **(B)** A schematic depiction of RiPP biosynthesis. Precursor peptide typically contains the leader peptide (in green) followed by the core peptide (in blue). Modifications of the core peptides (in brown) are guided by the leader peptides that interact with processing enzymes. Proteolytic release of the leader peptides then gives rise to mature RiPPs (in yellow).

in *Streptomyces* hosts after introduction of the constitutive ermE* promoter (Flinspach et al., 2014) and by a strong promoter (Hayashi et al., 2014), respectively (**Table 1**). Of note, the Link group constructed an expression system with two orthogonally inducible promoters to permit a separate control of the production and the export/immunity of lasso peptide MccJ25 in *E. coli* (**Table 1**, **Figure 3**). This elegant design enabled high-throughput screening of saturation mutagenesis libraries of the ring and β-hairpin tail regions of MccJ25 to obtain new insights to its structure-activity relationship (Pan and Link, 2011).

RBS Substitution to Optimize Translation Efficiency

A ribosomal binding site (RBS) is critical in initiating the translation of many downstream genes. Its efficiency depends on the core Shine-Dalgarno (SD) sequence, the surrounding secondary structure, and the spacing between the SD sequence and the start codon AUG. Upon translation initiation, the 3′-sequence of the 16S rRNA complementarily pairs with the SD sequence in the RBS. Over millions of years of evolution, microbes have created and utilized a diverse set of RBSs to control protein translation (Omotajo et al., 2015), which is also employed to regulate the production of secondary metabolites. As such, RBSs are an important component part of synthetic biology applications including the heterologous production of RiPPs and other families of natural products (Bai et al., 2015). For example, the incorporation of optimized *E. coli* RBSs has proven to be an efficient way to significantly increase the yields of multiple lasso peptides, including astexin−1,−2, and−3 (Maksimov et al., 2012; Maksimov and Link, 2013), capistruin (Pan et al., 2012), and caulosegnin (**Table 1**) (Hegemann et al., 2013a). In a more inclusive example, Hegemann et al. cloned the gene clusters of lasso peptides from various sources into the expression vector pET41a, and included a strong *E. coli* RBS in the intergenic region between their precursor gene(s) and the genes encoding

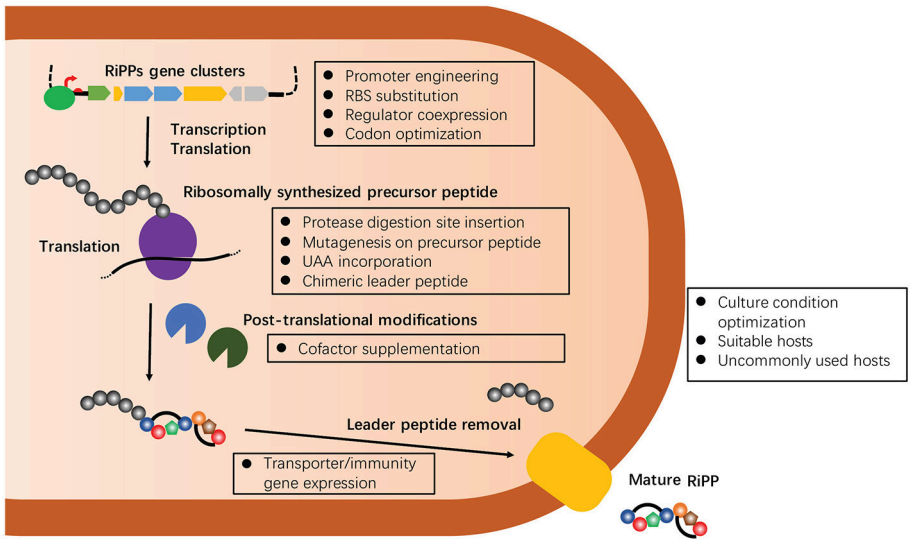

FIGURE 2 | A summary of multiple emerging strategies that target on manipulating individual proteins, pathways, metabolic flux or hosts to improve the success of heterologous expression of RiPPs. All of these strategies will be discussed below with select recent examples.

processing enzymes (**Table 1**; Hegemann et al., 2013b). This design increased the production yields of almost all expressed lasso peptides by 1.8- to 84.5-folds, although the deletion of extra precursor peptides might also contribute to the yield improvement in some cases (Hegemann et al., 2013b).

Optimization of the Catalytic Performance of Processing Enzymes

RiPP biosynthesis recruits a rapidly expanding list of functionally diverse enzymes to furnish structural and functional diversity (Arnison et al., 2013). The reactions of some RiPP biosynthetic enzymes require cofactors/co-substrates that may not be (or insufficiently) available in the surrogate host, leading to suboptimal production of targeted RiPPs. Therefore, optimal heterologous expression of RiPPs sometimes can be achieved by targeting cofactors/co-substrates of essential processing enzymes. For instance, NisB is a dehydratase involved in the biosynthesis of the food preservative nisin and its catalytic function requires glutamyl-tRNA^{Glu} as a co-substrate, uncommon to RiPP processing enzymes (Ortega et al., 2016). Accordingly, increasing the cellular availability of *Microbispora* sp. 107891 glutamyl-tRNA^{Glu} in *E. coli* was attempted to enhance the catalytic activity of MibB, a homolog of NisB involved in the biosynthesis of NAI-107. This study led to the production of NAI-107 analogs containing up to seven dehydrations, in contrast to nearly no dehydration when having no expressed *Microbispora* sp. 107891 glutamyl-tRNA^{Glu} (**Table 1**) (Ortega et al., 2016). In a more pronounced example, the Schmidt group found that the addition of cysteine (5–10 mM) to the culture media, along with minor process changes, increased the yield of cyanobactin patellins by 150-folds (**Table 1**; Tianero et al., 2016). It was proposed that sulfide derived from cysteine specifically modulates the substrate preference of cyanobactin processing enzymes, enabling post-translational control of product formation *in vivo*. Moreover, elevating the availability of the isoprene precursor, which is required by the pathway-specific prenyltransferase (Mcintosh et al., 2011), gave rise to an additional ∼18-fold increase of patellin yield in *E. coli* (**Table 1**).

Codon Optimization to Enhance Heterologous Expression

Due to the different abundance of tRNAs in various hosts, each organism has its own codon preference. Thus, codon optimization of biosynthetic genes proves to be a good strategy to achieve optimal heterologous expression. For example, the biosynthetic genes of geobacillin I, a nisin analog encoded by the thermophilic bacterium *Geobacillus thermodenitrificans* NG80-2, were codon-optimized before their introduction to *E. coli* for heterologous expression (Garg et al., 2012). Likewise, genes $cylL_L$, $cylL_S$, and *cylM* encoding the enterococcal cytolysin were synthesized with codon optimization for use in *E. coli* (Tang and Van Der Donk, 2013). Notably, in the heterologous expression of patellamides in *E. coli*, much lower yield was observed with vectors that were not codon-optimized (Schmidt et al., 2005).

Manipulation of Pathway-Specific Regulators

Despite the brevity of RiPP biosynthetic logic (**Figure 1B**), their gene clusters often encode components for precursor peptides, processing enzymes, resistance mechanism and regulators, the same as other families of natural products (e.g., polyketides and nonribosomal peptides) (Ortega and van Der Donk, 2016). Targeting any of these components, particularly the regulators of RiPP biosynthetic pathways, can favor the success of RiPP

TABLE 1 | Selected successful examples of heterologous expression of different RiPP families[a].

Subfamily of RiPPs	Natural products	Native host	Heterologous host
Bottromycin	Bottromycin (Huo et al., 2012)	*Streptomyces bottropensis*	*S. coelicolor* A3(2)
Bacteriocin	Bacteriocin enterocin A (EntA) (Jiménez et al., 2015)	*Enterococcus faecium*	*Lactobacillus* spp.
Cyanobactin	Patellamide A and C (Schmidt et al., 2005)	*Prochloron didemni*	*E. coli* BL21 (DE3)
Cyanobactin	Patellamide (Long et al., 2005)	*Prochloron didemni*	*E. coli* DH10B
Cyanobactin	Patellamide and ulithiacyclamide (Donia et al., 2006)	*Prochloron* spp.	*E. coli* Rosetta2 (DE3)
Cyanobactin	Trunkamide (Donia et al., 2008)	*Prochloron* spp.	*E. coli* TOP10
Cyanobactin	Anacyclamides (Leikoski et al., 2010)	*Anabaena* sp. 90	*E. coli* One Shot TOP10
Cyanobactin	Hexameric patellin (Tianero et al., 2012)	*Lissoclinum* sp.	*E. coli* TOP10
Cyanobactin	Trunkamide derivatives (Ruffner et al., 2015)	*Lissoclinum* sp.	*E. coli* 10-β
Cyanobactin	Telomestatin (Amagai et al., 2017)	*Streptomyces anulatus* 3533-SV4	*S. avermitilis* SUKA22
Cyclotide	Kalata B1 (Poon et al., 2018)	*Oldenlandia affinis*	*Nicotiana benthamiana*
Lanthipeptide I	Cinnamycin (Widdick et al., 2003)	*Streptomyces cinnamoneus* DSM 40005	*S. lividans* 1326
Lanthipeptide I	Microbisporicin (Foulston and Bibb, 2010)	*Microbispora corallina*	*Nonomuraea* sp. ATCC 39727
Lanthipeptide I	Geobacillin I (Garg et al., 2012)	*Geobacillus thermodenitrificans*	*E. coli* BL21 Gold
Lanthipeptide I	Modified gallidermin and nisin (Van Heel et al., 2013)	*Lactococcus lactis*	*L. lactis* NZ9000
Lanthipeptide I	Planosporicin (Sherwood et al., 2013)	*Planomonospora alba*	*Nonomuraea* sp. ATCC 39727
Lanthipeptide I	NAI-107 (Microbisporicin A1) (Ortega et al., 2016)	*Lactococcus lactis.*	*E. coli* BL21 Gold
Lanthipeptide II	Nukacin ISK-1 (Aso et al., 2004)	*Staphylococcus warneri* ISK-1.	*Lactococcus lactis* NZ9000
Lanthipeptide II	Prochlorosin 1.7, 2.11, 3.2, and 3.3 nisin (Shi et al., 2011)	*Prochlorococcus*	*E. coli* BL21 Gold
Lanthipeptide II	Cinnamycin (Ökesli et al., 2011)	*Streptomyces cinnamoneus* DSM 40005	*E. coli* BL21 Gold
Lanthipeptide II	Lichenicidin (Caetano et al., 2011a)	*Bacillus licheniformis*	*E. coli* BL21 Gold
Lanthipeptide II	Lichenicidin (Caetano et al., 2011b)	*Bacillus licheniformis*	*E. coli* BL21 Gold
Lanthipeptide II	Prochlorosin analogs (Tang and Van Der Donk, 2012)	*Prochlorococcus* MIT9313	*E. coli* BL21 Gold
Lanthipeptide II	Carnolysin (Lohans et al., 2014)	*Carnobacterium maltaromaticum* C2	*E. coli* BL21 Gold
Lanthipeptide II	Bovicin HJ50-like lantibiotics (Wang et al., 2014)	*Streptococcus bovis* HJ50	*E. coli* BL21 Gold
Lanthipeptide II	Lichenicidin (Kuthning et al., 2015)	*Bacillus licheniformis* I89	*E. coli* BL21 Gold
Lanthipeptide II	Pseudomycoicidin (Basi-Chipalu et al., 2015)	*Bacillus pseudomycoides*	*E. coli* C43
Lanthipeptide II	Lanthipeptides (Zhao and Van Der Donk, 2016)	*Ruminococcus flavefaciens*	*E. coli* BL21 Gold
Lanthipeptide IV	Streptocollin (Iftime et al., 2015)	*Streptomyces collinus* Tì 365	*S. coelicolor* M1146 and M1152
Lasso peptide	Capistruin (Knappe et al., 2008)	*Burkholderia thailandensis* E264	*E. coli* BL21 Gold
Lasso peptide	Microcin J25 (Pan and Link, 2011)	*E. coli* AY25	*E. coli* XL-1 Blue
Lasso peptide	Astexin-1 (Maksimov et al., 2012)	*Asticcacaulis excentricus*	*E. coli* BL21 Gold
Lasso peptide	Astexin-2 and−3 (Maksimov and Link, 2013)	*Asticcacaulis excentricus*	*E. coli* BL21 Gold
Lasso peptide	Burhizin, Caulonodin I, Caulonodin II, Caulonodin III, Rhodanodin, Rubrivinodin, Sphingonodin I, Sphingonodin II, Syanodin I, Sphingopyxin I, Sphingopyxin II, and Zucinodin (Hegemann et al., 2013b)	Multiple proteobacterial strains	*E. coli* BL21 Gold
Lasso peptide	Caulonodins IV to VII (Zimmermann et al., 2014)	*Caulobacter* sp. K31	*E. coli* BL21 Gold
Lasso peptide	MccJ25 UAA (Piscotta et al., 2015)	*E. coli* AY25	*E. coli* BL21 Gold
Lasso peptide	Benenodin-1 and−2 (Chekan et al., 2016)	*Asticcaucalis benevestitus*	*E. coli* BL21 Gold
Linaridin	Grisemycin (Claesen and Bibb, 2011)	*Streptomyces griseus* IFO 13350	*S. coelicolor* M1146
Microviridin	Microviridin J (Ziemert et al., 2008)	*Microcystis* UOWOCC MRC	*E. coli* Epi300
Microviridin	Microviridin L (Weiz et al., 2011)	*M. aeruginosa* NIES843	*E. coli* BL21
Omphalotin	Omphalotin A (Ramm et al., 2017)	*Omphalotus olearius*	*Pichia pastoris* GS115
Sactipeptides	Subtilosin A (Himes et al., 2016)	*B. subtilis* 168	*E. coli* BL21 (DE3)
Thiopeptide	Thiazolyl peptide GE37468 (Young and Walsh, 2011)	*Streptomyces* ATCC 55365	*S. lividans* TK24
Thiopeptide	Thiopeptide GE2270 (Tocchetti et al., 2013)	*Planobispora rosea*	*Nonomuraea* ATCC39727

(Continued)

TABLE 1 | Continued

Subfamily of RiPPs	Natural products	Native host	Heterologous host
Thiopeptide	Berninamycin (Malcolmson et al., 2013)	*Streptomyces bernensis* UC 5144	*S. lividans* TK24, *S. venezuelae* ATCC 10712
Thiopeptide	Silent thiopeptide biosynthetic Lactazoles gene cluster (Hayashi et al., 2014)	*Streptomyces lactacystinaeus* OM-6519	*S. lividans* TK23
Thiopeptide	Thiopeptide antibiotic GE2270 (Flinspach et al., 2014)	*Planobispora rosea* ATCC 53733	*S. coelicolor* M1146
Thioviridamide	Thioviridamide (Izawa et al., 2013)	*Streptomyces olivoviridis*	*S. lividans* TK23
Thioviridamide	JBIR-140 (Izumikawa et al., 2015)	*S. olivoviridis* OM13	*S. avermitilis* SUKA17
TOMM	Plantazolicin (Deane et al., 2013)	*Bacillus amyloliquefaciens* FZB42	*E. coli* BL21 (DE3)
TOMM	Microcin B (Metelev et al., 2013)	*Pseudomonas syringae*	*E. coli* BL21 (DE3)
Ustiloxin	Ustiloxin B (Ye et al., 2016)	*Ustilaginoidea virens*	*Aspergillus oryzae*

[a]*Entries were arranged first by the alphabetical order of the names of RiPP families and then chronically by the year of the publication.*

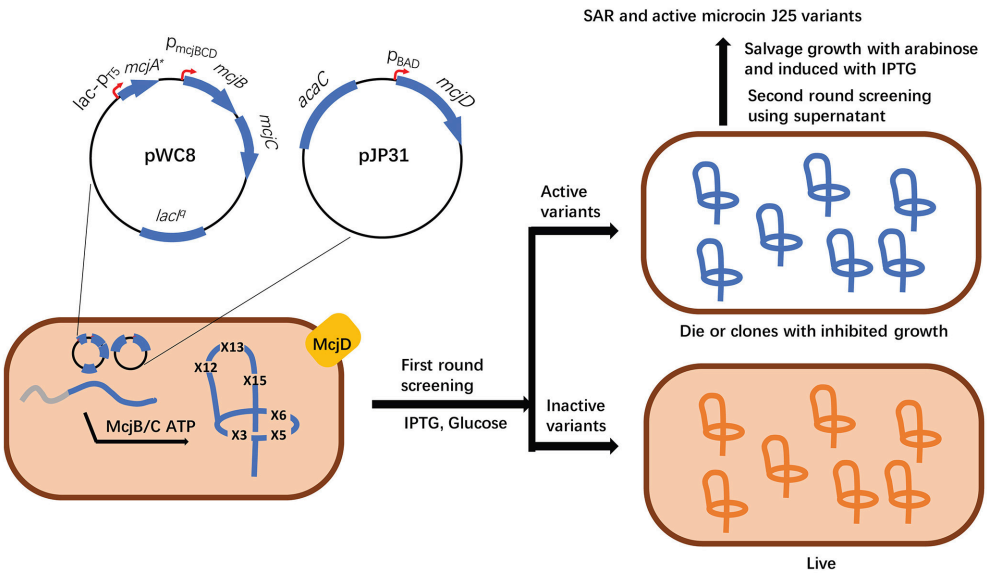

FIGURE 3 | High throughput discovery of functional microcin J25 variants with multiple amino acid substitutions was enabled by an orthogonally inducible system which separately controls the production and export/immunity of mature RiPPs. More specifically, the expression of the precursor gene *mcjA* and the transporter gene *mcjD* was independently induced by IPTG and arabinose, respectively. In the noninduced state, leaky expression leads to the low levels of both McjA and McjD (left). When IPTG and glucose are added, the expression of *mcjA* mutants is highly induced, but not *mcjD*, resulting in cytoplasmic accumulation of McjAs. If McjAs are processed into mature MccJ25 variants with antibacterial activity, accumulated lasso peptides will inhibit the growth of the host cell (top right). The poor growth of these cells will be salvaged by the addition of arabinose to overexpress McjD. By contrast, inactive MccJ25 variants will have no inhibitory effect on the cell growth (bottom right).

heterologous production. A comprehensive review on gene-regulatory mechanisms operating in RiPPs biosynthesis was recently reported elsewhere (Bartholomae et al., 2017). We highlighted here an example about the essentiality of a pathway-specific regulator to successful RiPP heterologous expression. The biosynthetic gene cluster of thiopeptide GE2270 (pbt) from *Planobispora rosea* ATCC 53733 previously failed to express the natural product in several *Streptomyces* hosts (**Table 1**; Tocchetti et al., 2013). In a recent report, Flinspach et al. revealed that the expression of PbtR, a TetR family of transcriptional regulator, is essential to the successful heterologous production in *S. coelicolor* M1146 (Flinspach et al., 2014).

Engineering Resistance Mechanisms to Improve RiPP Productivity

Natural products are known to possess biological activities that target organisms in the same environmental niches, thereby offering survival benefits (Behie et al., 2017). To avoid self-toxicity, the producers accordingly evolve many different types of resistance mechanisms (e.g., transporters, chemical modification and target modification), often embedded in the natural product gene clusters (Jia et al., 2017; Almabruk et al., 2018). Expectedly, resistance mechanisms can offer a way to regulate the production of natural products, including RiPPs. For example, the biosynthesis of the lantibiotic nisin in *Lactococcus*

lactis requires the dehydratase NisB, the cyclase NisC, the ABC-type transporter NisT, and the protease NisP, which together convert the precursor peptide NisA into the final product (Cheigh and Pyun, 2005). NisT forms a protein complex with NisB, C and P to effectively export bioactive nisin after its formation. Indeed, no secreted nisin was detected from the medium of a *L. lactis* mutant lacking the *nisT* gene, while the expression of *nisABCP* in this strain resulted in a considerable growth inhibition due to the intracellular accumulation of nisin (**Table 1**; Van Den Berg Van Saparoea et al., 2008). This example illustrates the necessity of a resistance mechanism to protect RiPP native producers. The same is likely true to surrogate hosts. For example, the ABC transporter MdnE was reported to be crucial for the successful production of a unique RiPP family, cyanobacterial tricyclic microviridins, in *E. coli* (**Table 1**; Weiz et al., 2011). In this case, MdnE might also act as a scaffold protein to guide the biosynthesis (Weiz et al., 2011). In another example, the multidrug transporter BotT of a bottromycin biosynthetic pathway is key to produce this antibiotic peptide in the surrogate host (Huo et al., 2012). Overexpression of the *botT* gene driven by a strong PermE* promoter in *S. coelicolor* host enhanced the production titer by 20 times compared to the control with the unmodified cluster. In addition to transporter genes, other resistance-imparting genes can also be used to boost the heterologous production of RiPPs. For instance, the heterologous production of the bacteriocin enterocin A (EntA) was accomplished by fusing a Sec-dependent signal peptide (SPusp45) with mature EntA and coexpressing the EntA immunity gene *entiA* (**Table 1**; Jiménez et al., 2015). EntiA protects the producing strain by forming a strong complex with the receptor protein, mannose phosphotransferase system, to avoid the toxicity. These manipulations led to a 4.9-fold higher production of EntA than the native producer (Jiménez et al., 2015).

Engineering Precursor Peptides to Produce RiPPs and Their Analogs

For the successful heterologous expression of RiPPs, one common hurdle is the lack of proper peptidases in the surrogate host to remove the leader peptide after finishing modifications on the core peptide (Bindman et al., 2015). Indeed, a number of RiPP gene clusters do not encode a protease dedicated to the removal of the leader peptide. The sequences of the linkers between the leader and core peptides also provide limiting information for the identification of such a protease from the genomes of native producers. To address this issue, the digestion site of a well-characterized, commercially available protease, such as GluC (Tang and Van Der Donk, 2012; Zhao and Van Der Donk, 2016;), trypsin (Himes et al., 2016), and C39 protease domain of the ABC transporter (Wang et al., 2014), can be engineered into the linker for the *in vitro* proteolytic release of the leader peptide from the matured precursor peptides isolated from heterologous hosts. As another approach, the van der Donk group genetically incorporated unnatural amino acids (UAAs) hydroxyl acids in the first position of a lanthipeptide by using a pyrrolysyl-tRNA synthetase-$tRNA^{Pyl}_{CUA}$ pair in *E. coli* (**Table 1**; Bindman et al., 2015). The installation of hydroxyl acid leads to an ester linkage between the leader and core peptides, which is readily cleavable by simple hydrolysis.

The majority of RiPPs precursor peptides comprise of the leader peptide region for the interactions with processing enzymes and the core peptide region that becomes the final products after chemical modification and proteolytic removal of the leader peptide (Arnison et al., 2013). The core region often carries multiple sequence variations that are tolerated by processing enzymes in modifications, providing opportunities to expand the chemical diversity of RiPPs. Indeed, genetic engineering of the core peptides of multiple RiPP families has led to impressive successes in exploring new chemical space for therapeutic applications. Two strategies have commonly been employed to diversify core peptide sequences, including single-site saturation mutagenesis (Young et al., 2012) and multiple-site sequence randomization (Ruffner et al., 2015; Yang et al., 2018). The first strategy is advantageous to screen small-size libraries but can miss desirable mutants that require multiple mutations on the core peptides. By contrast, the second strategy in principle explores the broadest chemical space covered by large libraries (e.g., 10^6-10^9 members), which is favored in drug discovery and development research. However, the success of this strategy depends on all three following factors, (1) the expression of all precursor peptide mutants in the host, (2) the proper processing of all mutants to generate large numbers of RiPP analogs, and (3) the high throughput screening methods to identify desirable compounds. In one recent example, Ruffner et al. employed the second strategy to randomly mutate the core peptide (TSIAPFC) of cyanobactin trunkamide (**Table 1**; Ruffner et al., 2015), whose processing enzymes are known to exhibit unusually relaxed sequence selectivity (Sardar et al., 2015). They prepared three double mutant libraries (XXIAPFC, TSXXPFC, and TSIXPXC) and a quadruple mutant library (XSXXPXC) in *E. coli* using the degenerate codon NNK. From the double mutant libraries (theoretically, 1,200 unique sequences in each library), they randomly screened a total of 460 clones, found 260 full-length precursor peptides, and detected 150 trunkamide analogs, giving a 33% success rate. The quadruple mutant library had the potential to produce 160,000 different sequences. The authors assessed the quality of this library by screening randomly picked 96 clones, found 65 full-length precursor peptides, and detected nine trunkamide analogs. The lower success rate (9.4%) of the quadruple mutant library may correlate with the selectivity of processing enzymes. In this regard, the van der Donk group recently leveraged the remarkable substrate tolerance of a lanthipeptide synthetase ProcM to generate a genetically encoded lanthipeptide library (Yang et al., 2018). They first randomized 10 positions of the core peptide of the precursor peptide ProcA2.8 (**Table 1**) (AACXXXXXSMPPSXXXXXC) using the NWY codon that encodes eight amino acids, leading to a 1.07×10^9 library. Limited by the transformation efficiency of *E. coli*, they obtained $\sim 10^6$ clones, 99.7% of which produced unique peptide sequences. Screening of 33 randomly selected clones led to identify 33 cyclized samples, illustrating the impressive versatility and substrate flexibility of ProcM. The authors then screened all 10^6 lanthipeptides using a cell survival-based high

throughput assay and identified one potent inhibitor of HIV p6 protein (Yang et al., 2018). In addition to the use of *E. coli* as a host to produce mutated RiPPs, both yeast display and phage display have recently been used to generate libraries of 10^6 lanthipeptides for screening for new bioactive analogs (Urban et al., 2017; Hetrick et al., 2018). These two well-characterized platforms can find more applications in expanding the chemical space of other RiPP families by the sequence randomization strategy.

In addition to 20 proteinogenic amino acids, a variety of UAAs can be used to expand the chemical diversity of RiPPs (Young and Schultz, 2010). This strategy has demonstrated its success with multiple RiPP families, including lantipeptide (Nagao et al., 2005; Oldach et al., 2012; Bindman et al., 2015; Kuthning et al., 2016; Lopatniuk et al., 2017; Zambaldo et al., 2017), lasso peptide (Piscotta et al., 2015), cyanobactin (Tianero et al., 2012), and sactipeptide (Himes et al., 2016). However, these unnatural RiPP analogs showed no significant improvement of their bioactivities possibly due to the relatively small extent of chemical expansion brought by a single UAA on a single position. However, coupled with the directed evolution of targeted core peptides, e.g., multiple-site randomization as described above, this strategy can generate new-to-nature RiPP analogs with enhanced structural and functional diversity.

RiPP precursor peptides physically separate their molecular recognition and catalysis sites for the processing by enzymes. Capitalizing on this distinct feature, a chimeric leader peptide strategy was recently developed to produce RiPP hybrids (Burkhart et al., 2017). Specifically, the leader peptides for the binding of thiazoline-forming cyclodehydratase, thioether-formation AlbA involved in the biosynthesis of sactipeptide, and lanthipeptide dehydratases NisB/C and ProcM were fused to allow sequential interactions with multiple processing enzymes of different RiPP families (**Figure 4**). As such, the engineered core peptides were received a combination of chemical transformations to produce unnatural peptide products, providing a generally applicable strategy to unlock the vast chemical space afforded by a variety of RiPP biosynthetic machinery (Burkhart et al., 2017).

MANIPULATION OF SURROGATE HOSTS FOR THE PRODUCTION OF RiPPs

Optimization of Culture Conditions

Screening a wide array of fermentation conditions, e.g., temperature, pH, shaking speed, nutrient levels, and trace metals, has routinely been practiced for the optimal production of target products. For example, Knappe et al. heterologously expressed the gene cluster of lasso peptide capistruin in *E. coli* and achieved a yield of 0.2 mg/L in the defined medium M20, which was 30% of its native producer *Burkholderia thailandensis* E264 in the same medium (**Table 1**) (Knappe et al., 2008). Surprisingly, no capistruin was produced when culturing transformed *E. coli* in commonly used LB medium. In another example, after testing a variety of conditions, the co-expression of Fe-S cluster biogenesis genes and lowered shaking speed together led to the significantly improved expression of subtilosin A in *E. coli* (Himes et al., 2016).

The Use of Suitable Hosts for the Production of RiPPs

An ideal host for the heterologous expression of natural products usually requires a clean background and high compatibility with the target biosynthetic gene cluster. More specifically, the ideal heterologous host would be able to supply abundant biosynthetic precursors from its primary metabolism while maintaining a relative clean secondary metabolic background, and also be capable of recognizing exogenous genetic parts, thus allowing access to the vast biosynthetic potential of the host. In this regard, *E. coli* has become one of the most popular heterologous hosts, and produced many RiPP families, e.g., cyanobactins, lantipeptides, lasso peptides, microviridins, and sactipeptides (Donia et al., 2006; Weiz et al., 2011; Metelev et al., 2013; Himes et al., 2016; Kuthning et al., 2016). On the other hand, the RiPP gene cluster from a high G+C producer is often expressed in a host with a relatively comparable genetic background. For example, the lantibiotic cinnamycin is produced by several *Streptomyces* strains and its gene cluster from *S. cinnamoneus cinnamoneus* DSM 40005 was successfully expressed in *S. lividans* to produce this peptidic antibiotic (Widdick et al., 2003). In another study, *S. lividans* TK23 and *S. avermitilis* SUKA17 were used as the hosts to produce thioviridamide (**Table 1**, **Figure 5A**; Izawa et al., 2013; Izumikawa et al., 2015). Interestingly, the expression of its gene cluster in *S. avermitilis* SUKA17 led to the production of a novel thioviridamide derivative, JBIR-140, further demonstrating the significant influence of a surrogate host on RiPP production.

In addition to the widely used hosts like *E. coli* and *Streptomyces* strains, several uncommon microorganisms have also been characterized as suitable RiPP heterologous hosts. With the consideration of available substrates and comparable genetic backgrounds, these heterologous hosts are often from the same family of the native producers of the target RiPPs. For example, when expressing the clusters of the lantibiotics microbisporicin and planosporicin and the thiopeptide GE2270 from *Microbispora coralline, Planomonospora alba* and *Planobispora rosea*, respectively, *Nonomuraea* sp. ATCC 39727, which is in the same family of the above native producers, acted as a viable host to produce corresponding RiPPs, but not multiple other tested *Streptomyces* strains (**Table 1**, **Figure 5B**; Foulston and Bibb, 2010; Sherwood et al., 2013; Tocchetti et al., 2013).

In recent years, fungal RiPPs have attracted increasing attentions given the availability of a number of fungal genomes in public domain (Hallen et al., 2007; Ding et al., 2016; Nagano et al., 2016; Ramm et al., 2017). To realize the chemical and functional potential of fungal RiPPs, their heterologous expression systems have to be established. In this regard, commonly used fungal strains can be initial targets in the development. Encouragingly, several biosynthetic genes of ustiloxin, the first filamentous fungal RiPP, were successfully expressed in *Aspergillus oryzae,* greatly facilitating the understanding of the macrocyclic formation and its entire biosynthetic pathway (**Table 1**, **Figure 5C**; Ye et al., 2016). In a more recent example, the partial reconstitution of the

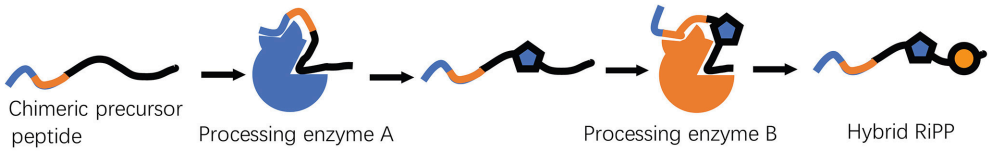

FIGURE 4 | A chimeric leader peptide strategy to produce unnatural RiPP hybrids. By properly designing the concatenated leader peptides, recognition and processing by multiple enzymes from unrelated RiPP pathways could be realized. By using this method, a thiazoline-forming cyclodehydratase was combined with biosynthetic enzymes from the sactipeptide and lanthipeptide families to create new-to-nature hybrid RiPPs, demonstrating the feasibility of the strategy.

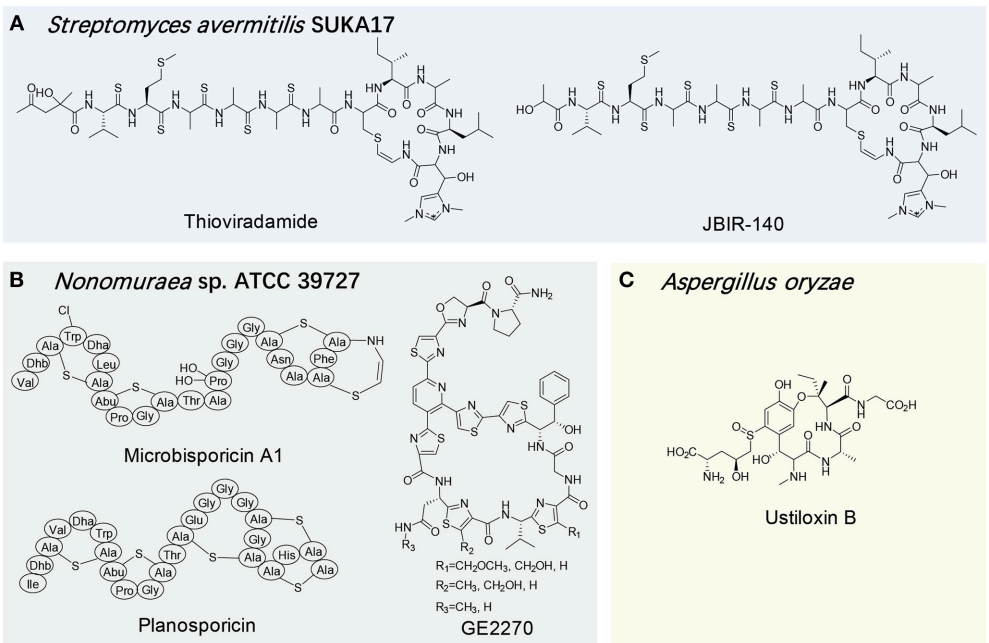

FIGURE 5 | Structures of select RiPPs produced by uncommon surrogate hosts exemplified by *Streptomyces avermitilis* SUKA17 **(A)**, *Nonomuraea sp.* ATCC 39727 **(B)** and *Aspergillus oryzae* **(C)**.

biosynthesis of one dodecapeptide omphalotin A, which is ribosomally produced by the basidiomycete *Omphalotus* olearius, was succeeded in *Pichia pastoris* strain GS115, but not *E. coli* (Ramm et al., 2017). This work further shed light on a novel biosynthesis mechanism for a RiPP in which a self-sacrificing enzyme, methyltransferase OphMA, bears its own precursor peptide.

Cyclotides are a family of plant-derived RiPPs that are characterized by a head-to-tail cyclic peptide backbone and a cystine knot arrangement of disulfide bonds. These peptidic compounds possess a wide range of bioactivities (e.g., protease inhibition, anti-microbials, and cytotoxicity) and are good carriers of other bioactive peptides, both of which are attractive to pharmaceutical research. Recently, the heterologous production of cyclotides were successfully achieved by co-expressing a select asparaginyl endoprotease and its precursor peptide *in planta*, using *Nicotiana benthamian*, tobacco, bush bean, lettuce, and canola as hosts (Poon et al., 2018). Interestingly, alternative strategies such as intein-mediated protein trans-splicing (Jagadish et al., 2013) and sortase-induced backbone cyclization (Stanger et al., 2014) have also been developed to produce cyclotides in bacterial and yeast expression systems, in which the asparaginyl endoprotease is not employed for the cyclization.

CONCLUSION AND FUTURE PERSPECTIVES

Harnessing the biosynthetic prowess of RiPPs via heterologous expression has witnessed several exciting advances in recent years. As described above, due to the conciseness of the biosynthetic route, the cloning and mobilization of the RiPP gene clusters typically do not constitute a major hurdle for the heterologous production of RiPPs. However, the functional expression of biosynthetic genes in surrogate hosts could be complicated by many less-predictable factors, such as the availability of protein cofactors, promoter recognition, product toxicity, protein–protein interaction, and imbalanced protein dosage. On the other hand, with *E. coli* and *Streptomyces*

strains serving as the most common hosts in the heterologous expression of RiPPs, the ever-increasing number of synthetic biology tools developed for these systems can be applied to overcome these challenges. In addition, *in vitro* characterization of RiPP biosynthesis and *in silico* prediction can be coupled to streamline and improve the outcomes of heterologous expression efforts. We are optimistic that a small set of highly developed hosts will be available as generally applicable platforms for rapid and robust sampling of the vast chemical space of RiPPs from bacteria, fungi, and even plants in future.

AUTHOR CONTRIBUTIONS

YZ, MC, SB, and YD planned, wrote and reviewed the manuscript.

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Engineering Heterologous Production of Salicylate Glucoside and Glycosylated Variants

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Salicylate 2-O-β-D-glucoside (SAG) is a plant-derived natural product with potential utility as both an anti-inflammatory and as a plant protectant compound. Heterologous biosynthesis of SAG has been established in *Escherichia coli* through metabolic engineering of the shikimate pathways and introduction of a heterologous biosynthetic step to allow a more directed route to the salicylate precursor. The final SAG compound resulted from the separate introduction of an *Arabidopsis thaliana* glucosyltransferase enzyme. In this study, a range of heterologous engineering parameters were varied (including biosynthetic pathway construction, expression plasmid, and *E. coli* strain) for the improvement of SAG specific production in conjunction with a system demonstrating improved plasmid stability. In addition, the glucoside moiety of SAG was systematically varied through the introduction of the heterologous oliose and olivose deoxysugar pathways. Production of analogs was observed for each newly constructed pathway, demonstrating biosynthetic diversification potential; however, production titers were reduced relative to the original SAG compound.

Keywords: salicylate, salicylate 2-O-β-D-glucoside, metabolic engineering, *E. coli*, analog

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INTRODUCTION

Plants have dedicated metabolism for the production of salicylate and a glycosylated version, salicylate 2-O-β-D-glucoside (SAG), which is often stored intracellularly until external stress is encountered (Vlot et al., 2009; Rivas-San Vicente and Plasencia, 2011). At which point, the reversion of SAG to salicylate allows the bioactivity of the latter compound to combat various biological threats to the plant system. Salicylate is also a central component of aspirin and, as such, SAG has the potential to possess similar anti-inflammatory properties.

These various bioactivities of SAG prompted us to explore its production through a heterologous bacterial host. Leveraging the knowledge and prior studies associated with engineering the shikimate pathway of *Escherichia coli* (Lin et al., 2014), we generated a production host supportive of high titer levels of salicylate (>1 g/L) (Ahmadi et al., 2016). This work included the introduction of an Irp9 salicylate synthase gene from *Yersinia enterocolitica*, which streamlined metabolism toward this precursor (**Figure 1**; Pelludat et al., 2003; Kerbarh et al., 2005). The introduction of a glucosyltransferase gene (*ugt74f1*) from *Arabidopsis thaliana* enabled conversion to the final SAG compound (Ahmadi et al., 2016).

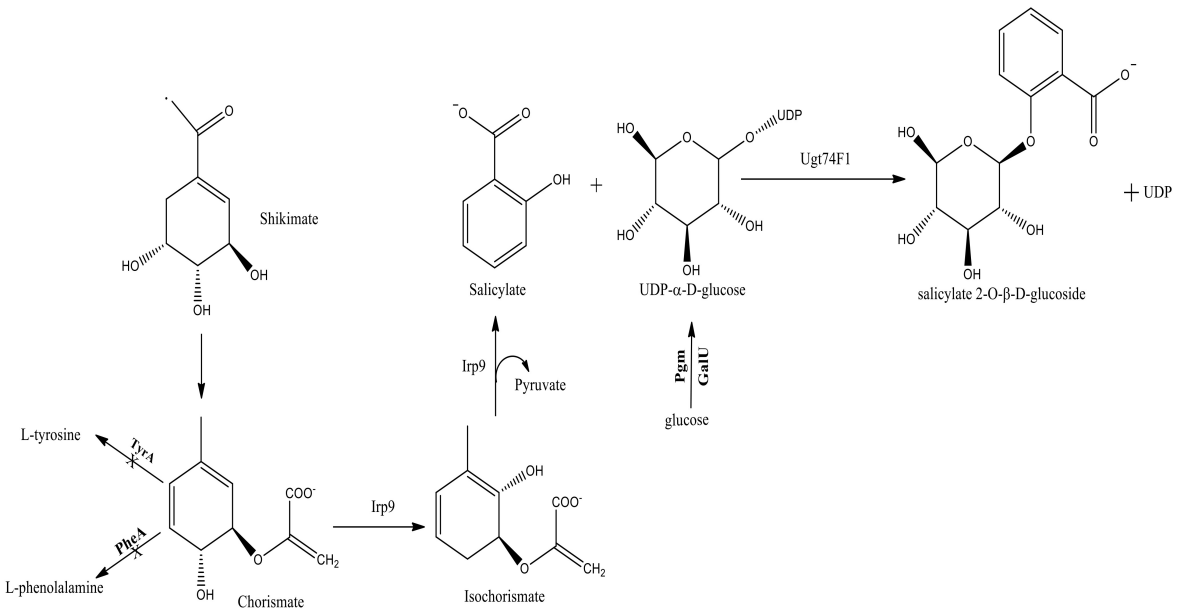

FIGURE 1 | The native and heterologous metabolic pathway for salicylate 2-O-β-D-glucoside (SAG) biosynthesis established within *E. coli*. The native TyrA, PheA, Pgm, and GalU steps were either deleted or over-expressed (bold); whereas the heterologous Irp9 and Ugt74F1 steps were introduced from *Yersinia enterocolitica* and *Arabidopsis thaliana*, respectively.

In the work presented herein, we were interested in improving SAG production through the application of various heterologous production parameters that spanned the *E. coli* production strain and several expression plasmids and associated components. In addition, based upon previous work by us and others toward glycodiversification of heterologous natural products, we tested the expanded analog potential of the original SAG compound through the systematic incorporation of two isomeric deoxysugar pathways.

MATERIALS AND METHODS

Plasmids and Strains

All cloning procedures were completed in *E. coli* TOP10 through which all recombinant plasmids were transferred and propagated. The *irp9* gene from *Y. enterocolitica* genomic DNA, the *pgm* and *galU* genes from *E. coli* K-12 MG1655, and a codon-optimized *ugt74F1* (salicylate glucosyltransferase gene) from *A. thaliana* were amplified by PCR with primers listed in **Supplementary Table S1**. Plasmids pMKA-41 and pGEX-UDP bearing these genes were constructed as described previously (Ahmadi et al., 2016) and used as the templates for the PCR reactions conducted in the current work. The PCR products were gel-purified and then digested with restriction enzyme pairs *Nhe*I/*Sal*I (for *irp9*), *Xba*I/*Sal*I (for *galU*), and *Nde*I/*Sal*I (for *pgm* and *ugt74F1*). Digested *irp9*, *pgm*, *galU*, and *ugt74F1* were separately ligated into similarly digested pET28a to yield pET28-*irp9*, pET28-*pgm*, pET28-*galU*, and pET28a-*ugt74F1*. Plasmid pET28-*pgm* was digested with *Xba*I/*Sal*I and the insert transferred to an *Spe*I/*Sal*I digested pET28-*galU* to yield pET28-*galU-pgm*. In the same way, the *Xba*I/*Sal*I digested *ugt74F1* fragment from pET28-*ugt74F1* was inserted into *Spe*I/*Sal*I digested pET28-*irp9* to construct pET28-*irp9-ugt74F1*. Plasmid pET28-*irp9-ugt74F1* was digested with *Xba*I/*Sal*I and the insert ligated to *Spe*I/*Sal*I digested pET28-*galU-pgm* to generate pRQS1. The pRQS1 *galU-pgm-irp9-ugt74F1* cassette featured in **Figure 2** was digested with *Nhe*I/*Bsi W*I and ligated into pETcoco-1 for subsequent digestion and transfer of the same cassette (using *Xba*I/*Sal*I) to pBAD33, yielding pRQS2 and pRQS3, respectively. A full list of plasmids and strains are presented in **Supplementary Table S2** and detailed plasmid maps are provided in **Supplementary Figures S1**, **S2**.

The double-knockout BW mutant (BW23) was constructed as described in previous work (Ahmadi et al., 2016) through deletion of *pheA* and *tyrA*, with these deletions improving both SA and SAG production. The λDE3 prophage was integrated into the BW23 chromosome through co-infection using a λDE3 Lysogenization Kit (Novagen), yielding BW23(DE3). By doing so, the new host is equipped with the λDE3 recombinant phage DNA encoding for the T7 RNA polymerase, therefore, allowing the expression of the *galU-pgm-irp9-ugt74F1* cassette though the T7 promotor within the pRQS1 and pRQS2 constructs. The pRSQ plasmids were transformed into corresponding strains through the standard heat-shock transformation protocol (**Supplementary Table S3**), and the resulting strains were stored as 20% glycerol stocks at −80°C.

The gene fragment for *irp9* was liberated from pET28-*irp9* via *Xba*I/*Sal*I digestion and then ligated into *Spe*I/*Sal*I digested pET28-*galU-pgm* to construct pET28-*galU-pgm-irp9*, which was then *Xba*I/*Sal*I digested and ligated into pET21c to generate pRQS4. Plasmids pGJZ1, 2, 3, and 4

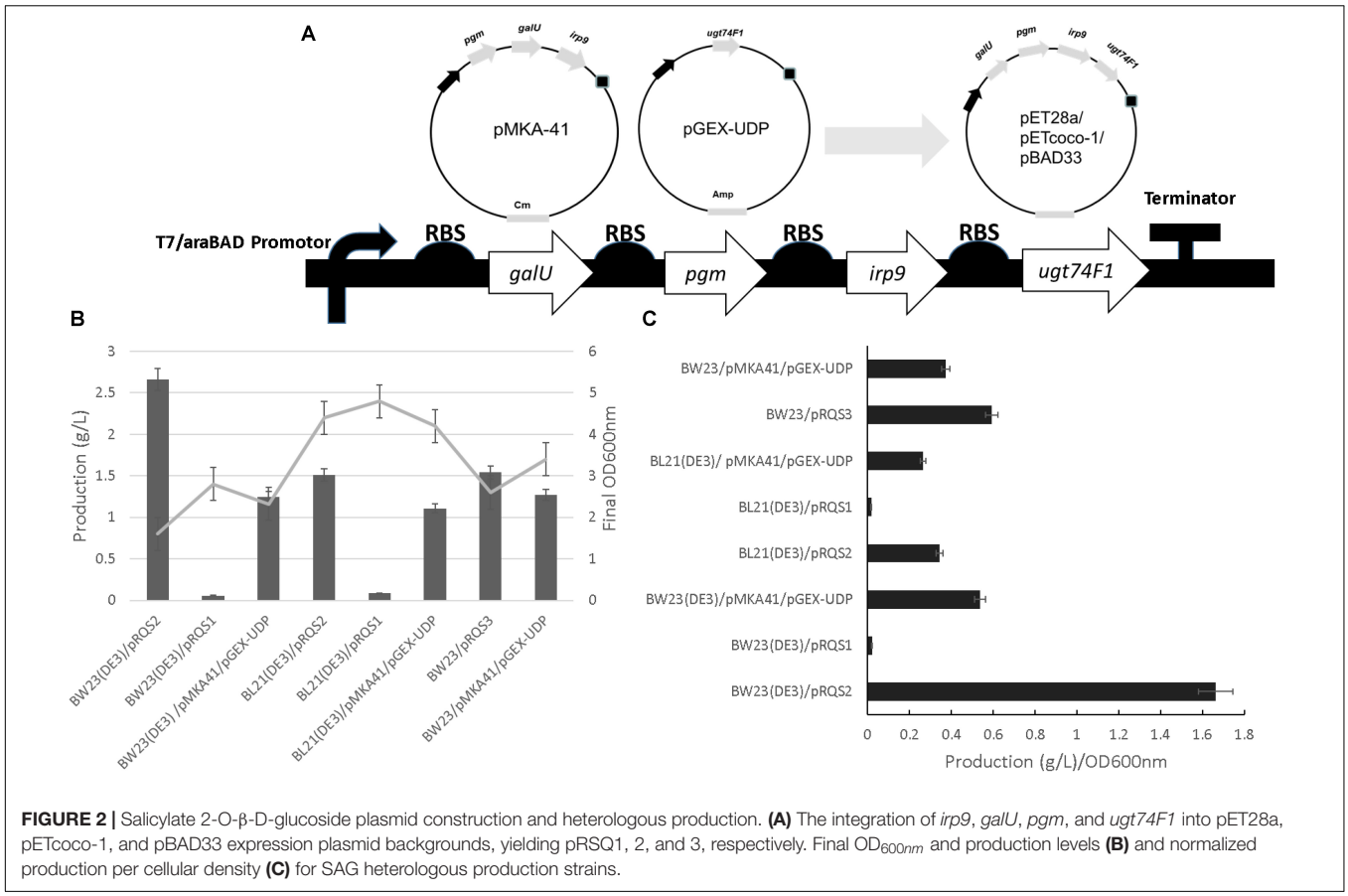

FIGURE 2 | Salicylate 2-O-β-D-glucoside plasmid construction and heterologous production. **(A)** The integration of *irp9*, *galU*, *pgm*, and *ugt74F1* into pET28a, pETcoco-1, and pBAD33 expression plasmid backgrounds, yielding pRSQ1, 2, and 3, respectively. Final OD_{600nm} and production levels **(B)** and normalized production per cellular density **(C)** for SAG heterologous production strains.

(Zhang et al., 2015), containing genes from the oleandomycin, chromomycin, and urdamycin A polyketide biosynthetic pathways, were used to produce two pairs of deoxysugar pathways for oliose and olivose. These four plasmids were integrated with codon-modified *urdGT* to construct pGJZ1-GT, pGJZ2-GT, pGJZ3-GT, and pGJZ4-GT (**Supplementary Figure S3**) which were, respectively, co-transferred with pRQS4 into BL21(DE3) (**Supplementary Figure S4** and **Supplementary Table S4**) to provide the complete biosynthetic pathways for SAG analogs.

Culture Conditions and Medium Components

The bacterial culture medium and associated chemical and analytical components were obtained from Sigma-Aldrich (St. Louis, MO, United States) or Thermo Fisher Scientific (Waltham, MA, United States). The DNA-manipulating agents, including restriction enzymes, T4 DNA ligase, Phusion High-Fidelity PCR Master Mix, and associated reagents were purchased from New England Biolabs (Ipswich, MA, United States). PCR primers (**Supplementary Table S1**) were obtained from Eurofins Genomics (Huntsville, AL, United States).

Respective glycerol stocks of producing strains from **Supplementary Table S3** were used to initiate overnight 3 mL cultures at 37°C with shaking in lysogeny broth (LB) medium prior to inoculating (1% v/v) 25 mL of M9Y medium which is formulated with (per liter): $Na_2HPO_4{\cdot}7H_2O$ (12.8 g); KH_2PO_4 (3 g); NaCl (0.5 g); NH_4Cl (1 g); yeast extract (1 g), glycerol (10 g), glucose (2.5 g), $MgSO_4{\cdot}7H_2O$ (246.5 mg), and $CaCl_2{\cdot}2H_2O$ (14.7 mg) supplemented with micronutrients including (per liter) vitamin B1 (2.0 mg), H_3BO_3 (1.25 mg), $NaMoO_4{\cdot}2H_2O$ (0.15 mg), $CoCl_2{\cdot}6H_2O$ (0.7 mg), $CuSO_4{\cdot}5H_2O$ (0.25 mg), MnCl2·4H_2O (1.6 mg), and $ZnSO_4{\cdot}7H_2O$ (0.3 mg). After inoculation, cultures were incubated at 30°C with shaking for 2 days with induction initiated using 200 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) and/or 3 mg/mL arabinose when the culture OD_{600nm} reached 0.4–0.6. As needed, plasmid selection in both liquid and solid medium was maintained with 100 mg/L ampicillin, 50 mg/L kanamycin, and 20 mg/L chloramphenicol.

Plasmid Stability Assay

Salicylate 2-O-β-D-glucoside producing strains were cultured in 25 mL production medium containing appropriate antibiotics at 37°C and 250 rpm until the OD_{600nm} reading reached 0.4–0.6. At this point, cultures were cooled to 22°C, induced for gene expression as described above, and incubated an additional 5 days. Plasmid stability analysis was completed on the first and fifth days after cultures were induced. At these time points, dilutions from each culture were spread evenly on LB agar plates

for incubation overnight at 37°C. Thirty colonies from each plate were selected and transferred to LB agar plates containing (1) combined antibiotics according to associated gene expression plasmids, (2) 1 mM IPTG, and (3) 1 mM IPTG + combined antibiotics as indicated in **Table 1**. Resulting colony development was then recorded, presented as a percentage of transferred colony growth on LB agar containing no antibiotics, and compared between strains.

Salicylate 2-O-β-D-glucoside (SAG) Production Quantification

Post-culture, 1 mL of acetone was added per 50 mL culture and a 1 mL sample was centrifuged. Supernatant (50 μL) was analyzed by HPLC as described previously (Dean et al., 2005). Briefly, SAG and associated analogs were quantified using a ZORBAX Eclipse XDB-C18 column connected to an Agilent 1100 system equipped with a diode array detector. Solvent A was 0.1% acetic acid in water, solvent B was methanol, and a flow rate of 1 mL/min was used across the following gradient: 5–20% solvent B over 10 min; 20–80% solvent B over 5 min; 80% solvent B maintained for 5 min; reset to 5% solvent B. An absorbance wavelength of 274 nm was used for both SAG and associated analog quantification (Ahmadi et al., 2016). Peak area quantification was conducted compared to a standard calibration curve of pure SAG (Toronto Research Chemicals, Toronto, ON, Canada).

LC-MS Analysis for SAG Analog Assessment

A 1 mL SAG analog culture sample was centrifuged and 50 μL of supernatant was used for analysis. LC-MS was performed using an API 3000 Triple Quad LC-MS with a Turbo Ion Spray source (PE Sciex) coupled with a Shimadzu Prominence LC system. Chromatography was performed through a Waters XTerra C18 column (5 mm, 2.1 mm × 250 mm) and MS analysis was conducted in positive ion mode. Following a 3 μL injection from the 50 μL sample, a linear gradient of 5–95% acetonitrile (balance water; both solutions containing 0.1% formic acid) was used for 20 min at a flow rate of 0.2 mL/min.

Statistical Evaluation

Data presented were generated from three independent experiments, and error bars represent standard deviation values.

RESULTS

As outlined in **Figure 2**, cellular parameters were engineered to improve specific SAG production. First, the production system designed previously for SAG formation [represented by plasmids pMKA-41 and pGEX-UDP (Ahmadi et al., 2016)] was organized into one operon introduced to three different expression plasmids (**Figure 2A**). Plasmids pETcoco-1 and pET28a allowed operon expression from the T7 promoter system coupled to a bacterial strain containing the T7 RNA polymerase (encoded within DE3 cellular variants). The pBAD33 plasmid featured expression driven by an arabinose inducible promoter system (Guzman et al., 1995). Plasmids were then introduced to strains BW23(DE3) and BL21(DE3) to accommodate the T7-based plasmids or BW23 for the pBAD33 plasmid system (also used for pMKA-41 and pGEX-UDP).

A production comparison revealed that the BW23(DE3)/pRSQ2 strain generated the best relative SAG levels based upon volumetric and specific titer comparisons (**Figures 2B,C** and **Supplementary Figure S5**). When comparing performance by either SAG titer or production per cell density, the BW23(DE3)/pRSQ2 strain demonstrated a twofold to fivefold improvement relative to the original BW23/pMKA-41/pGEX-UDP system. Of the new expression plasmids tested, pRSQ1 showed the lowest levels of SAG production. Of the production strains tested, the BW23(DE3) background, engineered to support SAG metabolic channeling and to accommodate the strong T7 promoter, demonstrated the best overall titers.

The plasmids used in this study included a low-copy option (pRSQ2; pETcoco-1 [OriV/S, 1–2 copies per cell]) and two medium copy options: pRSQ1 (pET28a [pBR322, ∼40 copies per cell]) and pRSQ3 (pBAD33 [pACYC184/p15A]). **Table 1** presents plasmid stability data for the associated SAG production strains. From this analysis, the RSQ2 plasmid shows the best

TABLE 1 | Plasmid stability comparison with antibiotic selection, IPTG induction, and combined IPTG induction and antibiotic selection.

	Antibiotic*		1 mM IPTG**		1 mM IPTG + Antibiotic***	
	Day 1	Day 5	Day 1	Day 5	Day 1	Day 5
BW23(DE3)/pRQS2	100	96.7	0	0	0	3.3
BW23(DE3)/pRQS1	100	100	10	36.7	6.7	66.7
BW23(DE3)/pMKA41/pGEX-UDP	100	83.3	3.3	16.7	0	13.3
BL21(DE3)/pRQS2	100	80	0	0	0	6.7
BL21(DE3)/pRQS1	100	66.7	6.7	26.7	0	56.7
BL21(DE3)/pMKA41/pGEX-UDP	100	53.3	0	6.7	3.3	20
BW23/pRQS3	100	90	0	10	0	10
BW23/pMKA41/pGEX-UDP	96.7	33.3	3.3	13.3	10	23.3

*Numbers represent percentage of transferred colony growth. *Cells carrying plasmid(s) are able to form colonies on plates containing corresponding antibiotics. **Those strains bearing no plasmid or mutants without the ability to express target genes can grow on plates with 1 mM IPTG. ***Those strains bearing plasmids but having lost the ability to express the target genes will form colonies on plates with both antibiotics and 1 mM IPTG.*

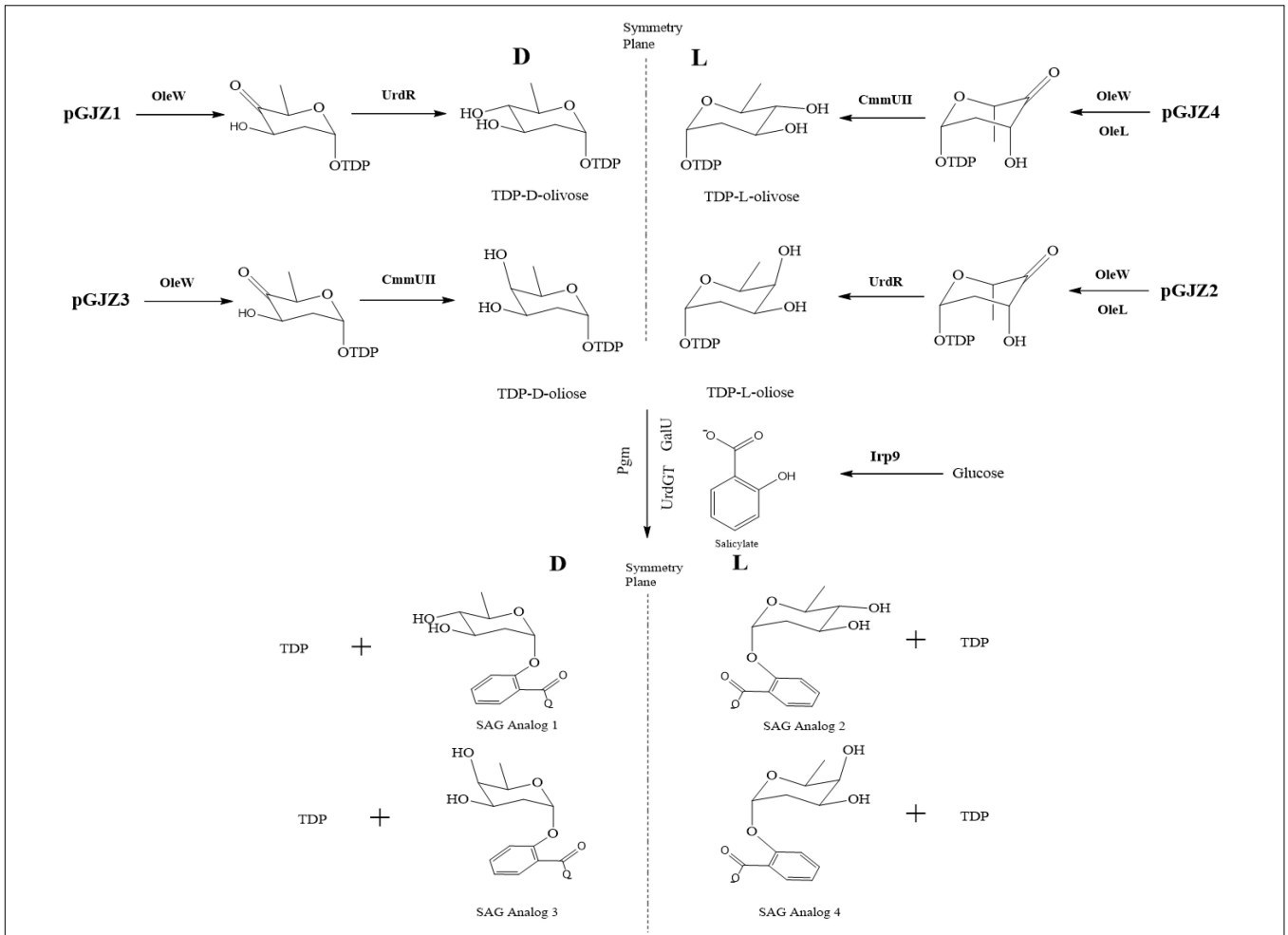

FIGURE 3 | Biosynthetic pathways for the production of SAG analogs. pGJZ plasmids 1–4 encode pairs of chiral-symmetric olivose and oliose deoxysugars to glycodiversify salicylate.

overall stability when tested for plasmid maintenance over time. The consolidated SAG biosynthetic pathways across plasmids pRSQ1-3 showed improved stability relative to the original dual expression plasmid system reliant upon pMKA-41 and pGEX-UDP. The same set of strains were also tested for stability when exposed to either IPTG induction or IPTG induction with antibiotic selection Novagen (1999). The plasmid stability data under these conditions indicate that, with the exception of pRSQ2, the newly constructed systems suffer from a combination of plasmid loss and mutant formation that results in lack of gene expression. From this perspective, the order of plasmid stability would be pRSQ2, pRSQ3, and pRSQ1.

Figure 3 outlines a schematic to test the flexibility of the SAG pathway to generate analogs resulting from alternative glycosylation patterning. In particular, isomeric variants of the oliose and olivose deoxysugars were tested for glycodiversification of the incoming salicylate precursor (Aguirrezabalaga et al., 2000). Initial analog production efforts with the Ugt74F1 salicylate glucosyltransferase resulted in minimal product formation (data not shown). As an alternative, the urdamycin system glycotransferase (UrdGT) was used due to previously observed flexibility in glycosylation patterning (Hoffmeister et al., 2000, 2003). Using HPLC and LC-MS analysis, data supporting analog formation were generated for each deoxysugar variant (**Figures 4A,B** and **Supplementary Figures S6**, **S7**). However, production levels were significantly reduced compared to those from the original SAG production systems (**Figure 2**).

DISCUSSION

A combination of metabolic engineering and gene expression design resulted in plasmids pRSQ1, 2, and 3. Each plasmid design consolidated genes needed for SAG biosynthesis. Furthermore, the plasmids featured different copy numbers and promoter strengths and, when combined with *E. coli* strains engineered to support gene expression and SAG production, allowed for a systematic evaluation of final product values. Production levels were best for strain BW23(DE3)/pRSQ2, which featured

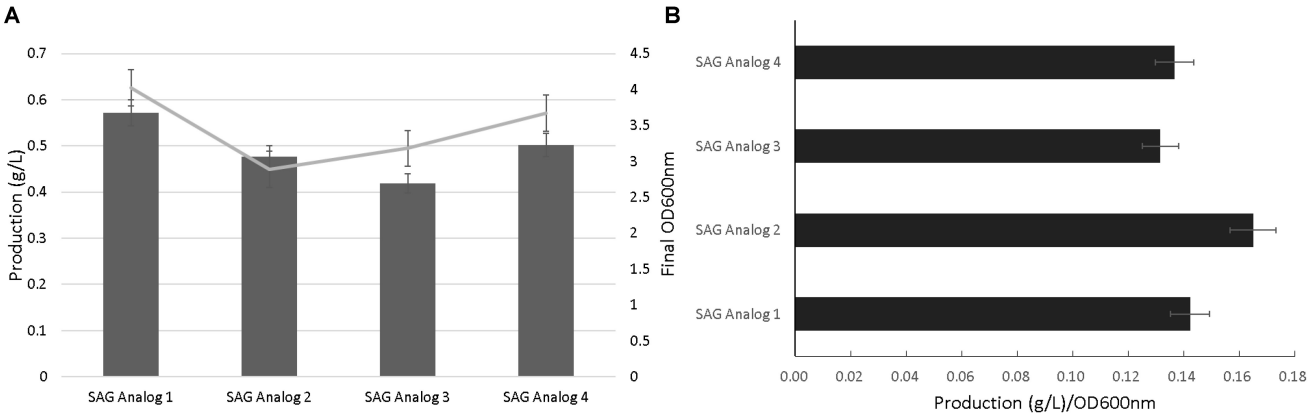

FIGURE 4 | Salicylate 2-O-β-D-glucoside analog production. Final OD_{600nm} and production levels **(A)** and normalized production per cellular density **(B)**.

a metabolically engineered strain to streamline carbon flow to SAG production and support the strong T7 promoter driving expression from pRSQ2. Of note, this particular expression plasmid was the lowest copy version of those tested. pRSQ1 featured T7-based gene expression but from a higher copy plasmid; pRSQ3 utilized an arabinose-inducible system within pBAD33 (similar to use previously with the control system BW23/pMDA-41/pGEX-UDP). This particular study focused only on SAG production as a function of plasmid biosynthetic pathway design (**Figure 2**); however, future engineering approaches, such as tuning biosynthetic steps via expression variation, will likely be needed to maximally drive complete salicylate to SAG formation (Ahmadi et al., 2016).

One likely contributor to the heightened production observed for pRSQ2 was incorporation of the highly stable OriV/S replication system (Shizuya et al., 1992; Monaco and Larin, 1994; Tao and Zhang, 1998). As such, even though the copy number for this plasmid was reduced, stability was improved as indicated within the results presented in **Table 1**. The strong T7 expression system likely compensated for the reduced copy number level (Golomb and Chamberlin, 1974; Studier and Moffatt, 1986); whereas, the higher copy number T7 system represented by pRSQ1 showed lower relative SAG production and higher plasmid loss. The pRSQ2 system has the added advantage (not tested in this study) of plasmid copy-up capability (Wild et al., 1996, 2001, 2002; Wild and Szybalski, 2004). Thus, there is the potential for further increased production through the stable maintenance of the pRSQ2 plasmid during the growth phase of the host system followed by induction of both SAG biosynthesis and plasmid amplification to spur subsequent product generation.

Our group and others have studied natural product glycodiversification, which offers a directed means of natural product structural variation (Thibodeaux et al., 2007, 2008; Williams et al., 2008; Jiang et al., 2013; Zhang et al., 2015; Fang et al., 2018). Given the glycosylated nature of SAG, we were interested if this product could also accommodate alternative sugar moieties. In conducting this work, we relied on a series of deoxysugar pathways previously incorporated into polyketide biosynthesis to generate erythromycin analogs (Zhang et al., 2015). However, contingent upon this strategy working is the flexibility of a glycosylation enzymatic step capable of accepting new substrate groups. Use of the original glucosyltransferas (Ugt74F1) resulted in minimal analog production. As a result, we turned to an alternative glycotransferase from the urdamycin biosynthetic pathway [recognized for substrate flexibility (Hoffmeister et al., 2002; Luzhetskyy et al., 2005) and the same source as some of the deoxysugar pathways genes], which resulted in the production level of the analogs presented in **Figure 4**. The ability for the urdamycin glycotransferase to accommodate novel SAG analogs supports the general theme of glycodiversification applied to this compound. As in the case of several previous examples of analogs produced as a result of biosynthetic pathway modification (Jiang and Pfeifer, 2013; Zhang et al., 2015), titer levels of the SAG analogs were significantly reduced compared to the original product, likely to do the new substrates limiting the catalytic activity of the glycosyltransferase. We also note that additional analytical work is needed to fully chemically characterize these new analogs. However, indication of novel analogs provides a basis for future studies to test potential variation in bioactivity in applications that range from inflammatory relief [as we have tested previously with the original SAG compound (Ahmadi et al., 2016)] or plant stress protection.

AUTHOR CONTRIBUTIONS

GZ and BP designed and supervised the study. RQ executed the experimental plan.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02241/full#supplementary-material

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Enhanced Biosynthesis of 2-Deoxy-*scyllo*-inosose in Metabolically Engineered *Bacillus subtilis* Recombinants

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2-Deoxy-*scyllo*-inosose (DOI) has been a valuable starting natural product for the manufacture of pharmaceuticals or chemical engineering resources such as pyranose catechol. DOI synthase, which uses glucose-6-phosphate (Glc6P) as a substrate for DOI biosynthesis, is indispensably involved in the initial stage of the biosynthesis of 2-deoxystreptamine-containing aminoglycoside antibiotics including butirosin, gentamicin, kanamycin, and tobramycin. A number of metabolically engineered recombinant strains of *Bacillus subtilis* were constructed here; either one or both genes *pgi* and *pgcA* that encode Glc6p isomerase and phosphoglucomutase, respectively, was (or were) disrupted in the sugar metabolic pathway of the host. After that, three different DOI synthase–encoding genes, which were artificially synthesized according to the codon preference of the *B. subtilis* host, were separately introduced into the engineered recombinants. The expression of a natural *btrC* gene, encoding DOI synthase in butirosin-producing *B. circulans*, in the heterologous host *B. subtilis* (BSDOI-2) generated approximately 2.3 g/L DOI, whereas expression of an artificially codon-optimized *tobC* gene, derived from tobramycin-producing *Streptomyces tenebrarius*, into the recombinant of *B. subtilis* (BSDOI-15) in which both genes *pgi* and *pgcA* are disrupted significantly enhanced the DOI titer: up to 37.2 g/L. Fed-batch fermentation by the BSDOI-15 recombinant using glycerol and glucose as a dual carbon source yielded the highest DOI titer (38.0 g/L). The development of engineered microbial cell factories empowered through convergence of metabolic engineering and synthetic biology should enable mass production of DOI. Thus, strain BSDOI-15 will surely be a useful contributor to the industrial manufacturing of various kinds of DOI-based pharmaceuticals and fine chemicals.

Keywords: 2-deoxy-*scyllo*-inosose, *Bacillus subtilis*, metabolic engineering, artificial gene, 2-deoxy-*scyllo*-inosose synthase

Abbreviations: DOI, 2-Deoxy-*scyllo*-inosose; Glc6P, glucose-6-phosphate.

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INTRODUCTION

Pyranose compounds have been produced in the traditional petrochemical sector from petroleum as a raw material. Most of these aromatic compounds including catechol and benzenoids are still being made from petroleum (Hansen and Frost, 2002; Baire et al., 2013), but because of limited petroleum reserves and worldwide regulations on carbon dioxide emissions, the development of environment-friendly and sustainable production processes using biomass (e.g., a fermentation process) is in demand.

2-Deoxy-*scyllo*-inosose (DOI) synthase, which uses Glc6P as a substrate when catalyzing the synthesis of pyranose compound DOI was first discovered as BtrC in *Bacillus circulans* that produces 2-deoxystreptamine-containing aminoglycoside butirosins (Kudo et al., 1999). This enzyme participates in the biosynthetic steps necessary for the core 2-deoxystreptamine scaffold (Llewellyn and Spencer, 2006; Park et al., 2013), and its catalytic product DOI has been broadly utilized as a starting material or a precursor of agrochemicals and pharmaceuticals (Hansen and Frost, 2002). Chemical synthesis of this DOI requires multistep reactions and hazardous and expensive metals, whereas the biosynthesis of DOI by DOI synthase allows for efficient synthesis in a single process. A method for producing DOI in a single enzymatic reaction was established (Takagi et al., 2006) in which a recombinant DOI synthase expressed in *Escherichia coli* is mixed with Glc6P. In addition, there was a report about a two-step enzymatic reaction that involves hexokinase and DOI synthase acting on D-glucose (Kakinuma et al., 2000). Furthermore, it was also reported that by the concentration of the enzymatic reactants followed by a reaction with hydrogen iodide under mild acidic conditions, DOI can be converted even to catechol (Suzuki et al., 2013).

Meanwhile, there was a publication concerning the biosynthesis of DOI via microbial fermentation of biomass-derived glucose by a recombinant strain of *E. coli*, into which a heterologous DOI synthase-encoding gene was introduced (Kogure et al., 2007). Accordingly, along with glucose, the rare and expensive sugar alcohol mannitol was also required as an extra carbon source for the support of microbial proliferation and growth of *E. coli*. In other words, having DOI synthase expressed in a wild-type strain of *E. coli* alone resulted in low productivity in terms of DOI, but high production of DOI (29.5 g/L), as reported, was achieved by simultaneous disruption of three genes essential for the primary metabolic pathway of *E. coli*: phosphoglucose isomerase (*pgi*), glucose 6-phosphate-1-dehydrogenase (*zwf*), and phosphoglucomutase (*pgm*). With all the metabolic pathways via which glucose can enter glycolysis eventually being blocked, mannitol that can be used in alternate glycolysis should be requisite for *E. coli* growth. In addition, a method for producing DOI from plant-derived ingredients, including sucrose as the less expensive carbon source than glucose, was reported (Tokuda et al., 2011). That is, a recombinant strain of *E. coli* that harbors both a sucrose-6-phosphate hydrolase (CscA) gene and a DOI synthase (BtrC) gene was created, through which a cost-effective and scalable fermentation process that produces DOI from sucrose as the major ingredient of molasses, was next developed. Moreover, a novel DOI synthase that shows relatively higher heat resistance and pH stability than the existing DOI synthases was also discovered (Konishi and Imazu, 2010). DOI synthase derived from the *Streptoalloteichus hindustanus* JCM3268 strain was also discovered (Hirayama et al., 2006). Most recently, there was a report on overexpression of the above *btrC* gene in the photoautotrophic cyanobacterium *Synechococcus elongatus*, resulting in 400 mg/L DOI photosynthesis without any need for a carbon source for the recombinant (Watanabe et al., 2018).

As mentioned above, DOI synthase is a crucial enzyme that is involved in the beginning of the biosynthesis of 2-deoxystreptamine-containing aminoglycoside antibiotics. Therefore, the gene encoding DOI synthase must be present typically within a number of biosynthetic gene clusters essential for the biosynthesis of relevant antibiotics such as gentamicin, kanamycin, and tobramycin: *genC* originating from gentamicin-producing *Micromonospora echinospora*, *kanC* from kanamycin-producing *Streptomyces kanamyceticus*, and *tobC* from tobramycin-producing *Streptomyces tenebrarius* (Park et al., 2013). Herein, three codon-optimized artificial genes ($genC_{opt}$, $kanC_{opt}$, and $tobC_{opt}$) were synthesized from the previously described *genC*, *kanC*, and *tobC* sequence templates (GenBank accession numbers AJ628149, AJ628422, and AJ810851, respectively) according to the codon usage preference of *B. subtilis* (**Figure 1**). Next, the glycolytic metabolic pathway in the heterologous host *B. subtilis* into which the above-mentioned artificial genes were being separately introduced was engineered; either one or both genes *pgi* and *pgcA*, which encode Glc6P isomerase and phosphoglucomutase, respectively, was (or were) disrupted in the primary metabolic pathway of the host (**Figure 1**). On the other hand, a gene *zwf* encodes Glc6P-1-dehydrogenase which plays its role in the branched routes from Glc6P as an initiator of oxidative pentose phosphate pathway (**Figure 1**). Based on the previous report (Zamboni et al., 2004), the *zwf* knockout mutant showed about 1.5-folds reduced growth rate compared with the wild-type strain. However, in case of other two genes (*pgi* and *pgcA*), there has been no report for the negative effect on the host growth. Therefore, in this study, we constructed Δpgi and ΔpgiΔpgcA knockout mutants, in which the *zwf* gene is still intact. The present study involves the design and construction of a number of metabolically engineered recombinant strains of *B. subtilis* into which several natural and artificial DOI synthase–encoding genes were introduced. This approach should ultimately provide a microbial cell factory platform that can produce DOI with a high titer and productivity, as compared to the existing technology.

MATERIALS AND METHODS

Construction of Bacterial Strains and Plasmids

Strain *B. subtilis* 168 (genotype: *trpC2*) served as a negative control (Belda et al., 2013). A gene-targeting method that does not require selection markers (Fabret et al., 2002) was employed for the construction of recombinant strains of *B. subtilis*, in which either one or both *pgi* (Glc6P isomerase

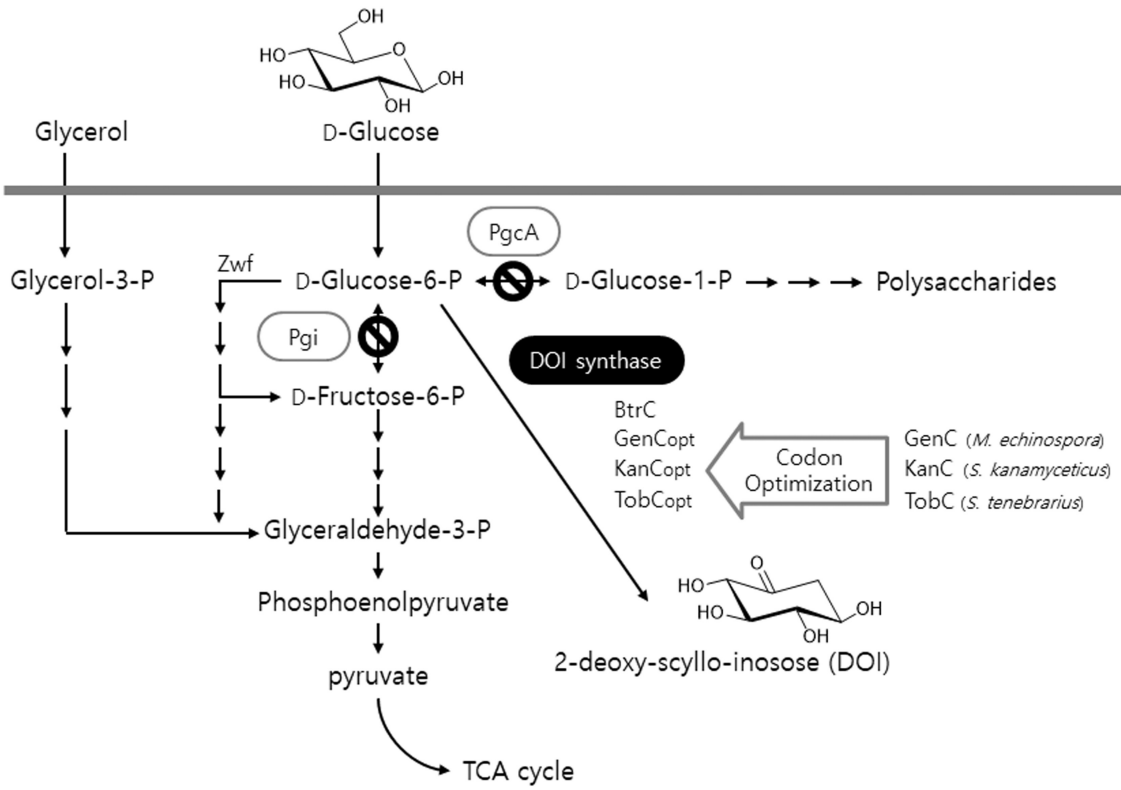

FIGURE 1 | An illustration of the production of DOI by the recombinant strains of *B. subtilis* via both engineering of the glycolytic metabolic pathway in the host and expression of codon-optimized artificial DOI synthases. TCA: tricarboxylic acid.

gene) and *pgcA* (phosphoglucomutase gene) involved in the glycolysis metabolic pathway (KEGG pathway ID bsu00010) was (or were) disrupted in the genome of the host. After transformation of integration plasmid pCU-pgiFB (phenotype: Cm^R [chloramphenicol resistance]) into *B. subtilis* strain 168, it was inserted into the *B. subtilis* genome via the first single-crossover recombination. The transformed recombinant Δpgi strain (genotype: *trpC2*Δ*pgi*, phenotype: Cm^R) was selected on a Luria-Bertani (LB; BD Biosciences, Sparks, MD, United States) solid plate medium supplemented with 5 μg/mL chloramphenicol (Sigma-Aldrich, St. Louis, MO, United States). After cultivation in the liquid LB medium for 18 h, the recombinants were counterselected on the minimal medium (MM) supplemented with 10 μM 5-fluorouracil (Sigma-Aldrich). Similarly, another integration plasmid, pCU-pgcAFB (phenotype: Cm^R), was introduced into the recombinant Δpgi strain to obtain the recombinant ΔpgiΔpgcA strain (genotype: *trpC2*Δ*pgi*Δ*pgcA*, phenotype: Cm^R) with both genes *pgi* and *pgcA* knocked out.

On the other hand, constitutive gene expression plasmid pHP13-P43 was constructed in the following manner. First, P_{43}, one of the promoters originating from *B. subtilis*, was amplified by PCR using a forward primer, P43-F (5′GGTA AAGCTTGCGGCTTCCTTGTAGAGCTCAG3′, underlined is a HindIII restriction enzyme cleavage site) and reverse primer P43-B (5′CTCTCTGCAGCATGTGTACATTCCTCTC3′, underlined is a PstI site). P_{43} promoter has been routinely utilized for the expression of heterologous gene in *B. subtilis*, as it is strong constitutive promoter (Song et al., 2016). After cleavage with both HindIII and PstI, the PCR products were ligated to *B. subtilis* expression plasmid pHP13 (*Bacillus* Genetic Stock Center, Columbus, OH, United States) that was digested with the same restriction enzymes, thus generating pHP13-P43 (genotype: P_{43}, phenotype: Cm^R, Em^R [erythromycin resistance]).

The above-mentioned integration plasmid pCU-pgiFB was constructed in the following manner. The upper and lower DNA fragments of the *pgi* gene within the *B. subtilis* genome, pgi-F and pgi-B, respectively, were amplified from the *B. subtilis* 168 strain genome as a template with a pair of primers: D-pgi-FU/L (5′CTAAACATGAACTGACAATTGAGGAAG3′) and D-pgi-BU/L (5′GAAGAAATATACAAGGTATCCAAAAGTATATG3′). Then, after fusion of these two DNA fragments, fusion PCR was performed with the D-pgi-FsnU/L primer. The PCR products were cleaved with restriction enzymes SphI and KpnI, and pCU-pgiFB was generated by ligating the amplicons to pCU (phenotype: Cm^R) that was digested with the same restriction enzymes. The other integration plasmid pCU-pgcAFB was produced by fusion PCR with equivalent primers (D-pgcA-FU/L: 5′TTAAGTTTATCGGTGAAAAGATTAAGGAATAC3′ and D-pgcA-BU/L: 5′AAAACCATATTCGTTAAAGAGATTGAT GAG3′) and the same restriction enzyme sites.

The expression plasmid pHP13-P43-BtrC used for introducing *btrC*, a *B. circulans*-derived DOI synthase encoding gene, into *B. subtilis* strain 168 was assembled as follows. In other words, the genomic DNA of *B. circulans* NRRL B3312 was prepared as a template, and then subjected to PCR with forward primer BtrC-F (5′GTGGGTACCGAGGTTAAA CATGACTAAAC3′, underlined is a KpnI site) and reverse primer BtrC-B (5′-CTCCTGCAGTTGTTATCGTGGATT AAATAATGG3′, underlined is a PstI site). After cleavage by both KpnI and PstI, the PCR products were ligated to expression plasmid pHP13-P43 that was digested with the same restriction enzymes, thereby yielding pHP13-P43-BtrC (genotype: P_{43}-*btrC*, phenotype: Cm^R, EM^R).

To amplify the expression of DOI synthase–encoding genes derived from microbes other than *Bacillus* (*genC* [*Micromonospora echinospora* DSM 43036, GenBank accession number AJ628149], *kanC* [*Streptomyces kanamyceticus* DSM 40500, GenBank AJ628422], and *tobC* [*Streptomyces* sp. DSM 40477, GenBank AJ810851]), the codon-optimized gene fragments (*genC*$_{opt}$, *kanC*$_{opt}$, and *tobC*$_{opt}$) were designed and artificially synthesized by CosmoGenetech (Seoul, Korea) according to the codon usage preference of the heterologous host *(B. subtilis)*. To all the codon-optimized gene fragments, we added a KpnI restriction site upstream of the ribosome-binding site and a PstI restriction site downstream of the termination codon, respectively. Three different artificial genes were treated with KpnI and PstI, and then ligated into plasmid pHP13-P43 that had been digested with the same restriction enzymes, thus generating pHP13-P43-GenC$_{opt}$ (genotype: P_{43}-*genC*$_{opt}$, phenotype: Cm^R, EM^R), pHP13-P43-KanC$_{opt}$ (genotype: P_{43}-*kanC*$_{opt}$), and pHP13-P43-TobC$_{opt}$ (genotype: P_{43}-*tobC*$_{opt}$), respectively.

Gene manipulation was performed by standard techniques and transformation of both *E. coli* and *B. subtilis* was carried out by heat shock transformation and the natural competent transformation (Green and Sambrook, 2012). Antibiotics were added to the medium of recombinant strains at appropriate concentrations (kanamycin 5 μg/mL, ampicillin 100 μg/mL, and chloramphenicol 5 μg/mL; all from Sigma-Aldrich). All amplicons were routinely verified by sequencing.

Shake-Flask Fermentation by the Recombinant Strains of *B. subtilis*

Unless specified otherwise, *E. coli* and recombinant *B. subtilis* strains were cultivated in the LB medium at 37°C. To examine the growth of the recombinant strains and their DOI productivity, 20 mL of the 2YTG liquid medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl, 3% glucose, all as w/v) was placed into a 250 mL baffled Erlenmeyer flask. After that, each recombinant strain was inoculated and cultivated for 12 h under the conditions mentioned above. A slightly modified 2YTG medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl, 3% glucose, 2% glycerol; w/v) which contains, as another carbon source, 2% of glycerol in addition to glucose was prepared for a fed-batch fermentation process. After inoculation into 250 mL of the shake-flask fermentation medium in a 1 L baffled Erlenmeyer flask, each recombinant was cultivated for up to 60 h at 37°C by reciprocal shaking at 200 rpm. Meanwhile, after 30 h of this shake-flask fermentation, 5 mL of a glucose solution (0.5 g/mL) that had been prepared under sterile conditions was added to the fed-batch culture to test whether the DOI productivity increases.

Growth Curves of the Recombinant Strains and Analyses of Their DOI Titers

During the entire 60 h of fed-batch fermentation, an aliquot (2 mL) of fermentation broth was collected every 10 h to construct the growth curve in the following manner: 1 mL of serially diluted broth was placed into the UV-Vis spectrophotometer (Shimadzu, Japan) with absorbance measured at 680 nm. After refrigerated centrifugation of the same sample for 3 min at 5000 rpm (or ∼8000 × *g*), the precipitated cell debris were freeze-dried and then weighed. The linear curve of correlation between the dry cell weights and absorbance values was drawn, with which the dry cell weights of the recombinant strains of *B. subtilis* were determined during the fed-batch fermentation.

On the other hand, quantification of DOI produced via the fed-batch fermentation by the recombinants was performed by means of a modified version of the procedures described in other reports (Yamauchi and Kakinuma, 1992; Kharel et al., 2004) as follows: 100 μL of the supernatant obtained by the above-mentioned refrigerated centrifugation was mixed with 300 μL of an aqueous methanol solvent (methanol/water at 1:2, v/v) together with 40 μL of *O*-(4-nitrobenzyl) hydroxylamine hydrochloride (Sigma-Aldrich), 35 mg/mL. The oxime adducts of DOI were prepared via the reaction for 30 min in a water bath set to 60°C (Kharel et al., 2004), and then the reactants were evaporated to dryness at room temperature by vacuum centrifugation. The DOI derivatives were reconstituted in 100 μL of methanol, and an aliquot (25 μL) was subjected to ultra high-performance liquid chromatography (UPLC) with electrospray ionization (ESI) and ion trap tandem mass spectrometry (MS/MS) analysis. The Spectra system P1000XR UPLC consists of a pump (Thermo Finnigan, San Jose, CA, United States) and a Spectra Series AS3000 autosampler (Thermo Finnigan, 20 μL loop). Isocratic chromatography was conducted on an Acquity CSH C_{18} (Waters, Milford, MA, United States) reversed-phase column at a flow rate of 120 μL/min of the mobile phase (acetonitrile/methanol/water/formic acid 1:1.5:8:0.002, v/v/v/v). The column effluent was directed to an LCQ ion trap mass spectrometer (Thermo Finnigan), operated in positive ion mode. The mass transition specific to DOI oxime adducts was *m/z* 315.3 > 164.3 (**Figure 2A**). Quantification of DOI produced during the fed-batch fermentation by each recombinant was carried out according to the calibration equation obtained from the correlation between the chromatographic peak areas and the concentration of the oxime adducts of an authentic DOI standard (GeneChem Inc., Daejeon, Korea). Each fermentation broth sample was also centrifuged, and then the supernatant of the sample was processed using two kinds of commercially available kits such as the D-glucose HK assay kit (Megazyme International Ltd., Wicklow, Ireland) and glycerol determination

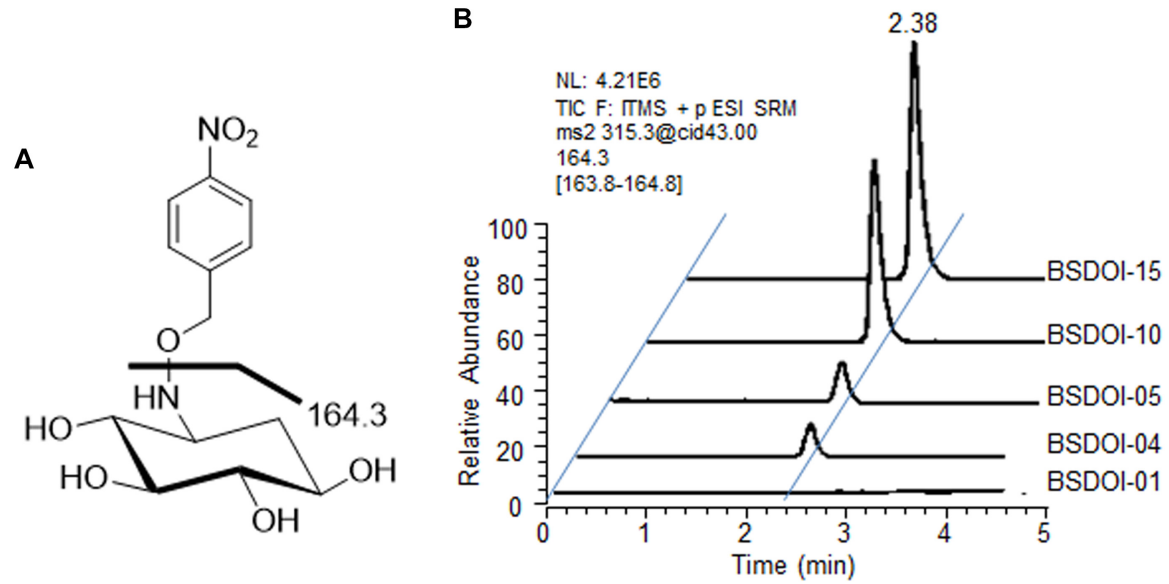

FIGURE 2 | UPLC-ESI-MS/MS analysis of DOI produced by the recombinant strains of *B. subtilis*. **(A)** An ESI-MS/MS fragmentation pattern (*m/z* 315.3 > 164.3) of a DOI oxime derivative produced by the recombinants described herein. **(B)** A typical UPLC-ESI-MS/MS chromatogram for quantification of the DOI titer in the shake-flask fermentation broths of the recombinants.

kit (Sigma-Aldrich) to determine the residual concentration of glucose and glycerol that had been added as carbon sources during fed-batch fermentation.

Growth curves and the DOI titer of recombinant strains of *B. subtilis*, together with the remaining glucose and glycerol content in the fed-batch fermentation process, were presented by averaging the results in quadruplicate.

RESULTS AND DISCUSSION

Comparison of the DOI Titer Produced by the Recombinants That Express a Heterologous DOI Synthase

Among the recombinant *B. subtilis* strains that carried out 60 h of shake-flask fermentation as described before, DOI productivity of four recombinant strains that expressed DOI synthase solely without further metabolic pathway engineering was determined and compared with that of strain BSDOI-01 as a negative control (**Table 1**). When a *btrC* gene that encodes the DOI synthase from *B. circulans* was expressed in the *B. subtilis* host (BSDOI-02), an average of 2.3 g/L DOI was produced. A recent publication revealed that DOI production of 1.5 g/L can be achieved through expression of the identical *btrC* gene in *E. coli* as a host (Kogure et al., 2007). Meanwhile, when an artificial gene—$genC_{opt}$ synthesized on the basis of a previously described *genC* sequence template—was introduced into the host (BSDOI-03), DOI titer in the shake-flask fermentation broth remained at 0.8 g/L on average, showing a failure of the enhancement of DOI productivity through $genC_{opt}$ expression. In contrast, heterologous expression of $kanC_{opt}$ and $tobC_{opt}$, both of which originate from the *Streptomyces* genus, in *B. subtilis* hosts (BSDOI-04 and BSDOI-05) increased the DOI titer up to 3.4 and 3.6 g/L, respectively (**Figure 2B**). In particular, the GC contents of two codon-optimized artificial genes $kanC_{opt}$ and $tobC_{opt}$ (from *Streptomyces*) are 52 and 59% respectively, whereas that of $genC_{opt}$ (from *Micromonospora*) is 68% even being synthesized according to the codon-usage preference in *B. subtilis* host, suggesting the negative effect of high GC-content onto the gene expression in heterologous host. Therefore, we found that the expression of $kanC_{opt}$ or $tobC_{opt}$ improved the DOI titer as compared with *btrC*, which has been in common use according to the existing publications (Kogure et al., 2007; Tokuda et al., 2011; Watanabe et al., 2018). Thus, it was suggested that the expression of DOI synthases (orthologous to different species) that are involved in the same catalytic reaction could yield diverse DOI titers, whereas it was found that an improved DOI titer, as compared to the results from preceding studies (Kogure et al., 2007; Tokuda et al., 2011), could be achieved through the expression of artificially synthesized genes based on synthetic biology that takes into account codon usage preferences of the recombinant strains.

Comparison of DOI Titers Produced by the Metabolically Engineered Recombinant Strains That Express *btrC*

DOI productivity of *btrC*-expressing recombinant strains (BSDOI-07 and BSDOI-12), in which either one or both *pgi* (Glc6P isomerase gene) and *pgcA* (phosphoglucomutase gene) involved in the glycolysis metabolic pathway was (or were) disrupted in the genome of *B. subtilis* as a host, was compared with that of the *btrC*-expressing BSDOI-02. BSDOI-07 represents

the *btrC*-expressing Δpgi strain, whereas BSDOI-12 denotes the *btrC*-expressing ΔpgiΔpgcA strain of *B. subtilis*. The resulting strains BSDOI-07 and BSDOI-12 showed average DOI productivity of 16.7 and 20.7 g/L, respectively, which was more than seven- and ninefold higher than the DOI productivity (2.3 g/L) of the control (BSDOI-02), which did not undergo a metabolic pathway modification (**Table 1**). In other words, when two kinds of enzymes (such as Glc6P isomerase and phosphoglucomutase that utilize Glc6P as the common substrate within the branched glycolytic metabolic pathway) were deleted in the host, intracellular accumulation of Glc6P as a typical substrate of DOI synthase was induced, thus leading to significantly elevated DOI productivity through catalysis by the BtrC enzyme. Moreover, the aforementioned three kinds of recombinant strains did not show noticeable differences in cell growth during the shake-flask fermentation process.

Comparison of DOI Titers Among the Metabolically Engineered Recombinant Strains That Express Codon-Optimized Artificial Genes

A natural *btrC* gene introduced into the Δpgi strain (BSDOI-7) as described above was replaced by three different kinds of codon-optimized artificial genes such as $genC_{opt}$, $kanC_{opt}$, and $tobC_{opt}$, yielding recombinant strains BSDOI-08 to BSDOI-10. Moreover, a similar gene replacement process was carried out in the ΔpgiΔpgcA strain (BSDOI-12), thereby generating recombinant strains BSDOI-11 to BSDOI-13. The DOI titer produced by the recombinants was compared with that of two recombinant strains: BSDOI-07 and BSDOI-12. At first, among the recombinant strains with single deletion of the *pgi* gene, additional expression of artificial gene $genC_{opt}$ showed a noticeably decreased DOI titer (5.5 g/L) relative to the *btrC*-expressing BSDOI-07 strain (**Table 1**). Nonetheless, replacement of *btrC* with other codon-optimized DOI synthases ($kanC_{opt}$ and $tobC_{opt}$) showed an improved DOI titer (average of 22.5 and 24.2 g/L, respectively) as compared to the control (average of 16.7 g/L). Similarly, recombinant strains with double deletion of genes *pgi* and *pgcA* showed a similar pattern of DOI titer; in particular, expression of artificial gene $tobC_{opt}$ in the above-mentioned recombinant host further increased DOI production to 37.2 g/L, on average (**Table 1** and **Figure 2**). Judging by the results above, engineering the glycolytic metabolic pathway in the host enables intracellular accumulation of Glc6P, and furthermore, the highest DOI titer and productivity were accomplished by means of artificial genes, as compared to other studies (Kakinuma et al., 2000; Kogure et al., 2007; Tokuda et al., 2011) that employed enzymatic reactions or an engineered recombinant strain of *E. coli*. Therefore, by combining the synthetic biology approach that spurs DOI biosynthesis in a metabolically engineered heterologous host *E. coli* strain, a maximum DOI production of 37.2 g/L was achieved. Hence these results highlight the above-mentioned 16-fold higher DOI titer compared with that of the *btrC*-expressing recombinant.

TABLE 1 | 2-Deoxy-*scyllo*-inosose titers shown by the recombinant *B. subtilis* strains during fed-batch fermentation.

Strain	Genotype specification	DOI (g/L)
BSDOI-01	*Bacillus subtilis* 168 [BS] + pHC13-P_{43}	Not detectable
BSDOI-02	BS + pHC13- P_{43}-*btrC*	2.3 ± 0.2
BSDOI-03	BS + pHC13- P_{43}-$genC_{opt}$	0.8 ± 0.2
BSDOI-04	BS + pHC13- P_{43}-$kanC_{opt}$	3.4 ± 0.3
BSDOI-05	BS + pHC13- P_{43}-$tobC_{opt}$	3.6 ± 0.2
BSDOI-06	BSΔpgi	Not detectable
BSDOI-07	BSΔpgi + pHC13- P_{43}-*btrC*	16.7 ± 0.7
BSDOI-08	BSΔpgi + pHC13- P_{43}-$genC_{opt}$	5.5 ± 0.7
BSDOI-09	BSΔpgi + pHC13- P_{43}-$kanC_{opt}$	22.5 ± 2.0
BSDOI-10	BSΔpgi + pHC13- P_{43}-$tobC_{opt}$	24.2 ± 1.4
BSDOI-11	BSΔpgiΔpgcA	Not detectable
BSDOI-12	BSΔpgiΔpgcA + pHC13- P_{43}-*btrC*	20.7 ± 1.1
BSDOI-13	BSΔpgiΔpgcA + pHC13- P_{43}-$genC_{opt}$	6.4 ± 1.0
BSDOI-14	BSΔpgiΔpgcA + pHC13- P_{43}-$kanC_{opt}$	29.0 ± 2.9
BSDOI-15	BSΔpgiΔpgcA + pHC13- P_{43}-$tobC_{opt}$	37.2 ± 2.4

Fed-Batch Fermentation by the BSDOI-15 Recombinant Strain

To examine time courses of cell growth and DOI production during the fermentation driven by a resultant engineered BSDOI-15 cell factory, the culture broth was collected every 10 h. The dry cell weight and the fermentation profiles of glucose and glycerol, together with the DOI titer were quantitatively determined (**Figure 3** and **Supplementary Figures 1**, **2**). Bacterial cell growth showed a typical fed-batch culture pattern, indicating that besides glucose, glycerol can serve as an extra carbon source for the growth of these DOI-producing cell factories. In fed-batch fermentation by the BSDOI-15 recombinant where glucose was added after 30 h fermentation, the profile of DOI production appears to closely correlate with that of glucose consumed. However, by the first 20 h of fed-batch fermentation, the concentration of glucose consumed was about 17.6 g/L, whereas DOI production was up to 27.9 g/L. Ideally, there should be one to one correspondence between the both concentrations. These discrepancies in reciprocal correlations between the glucose consumption and DOI production within 20 h fermentation might be due to the usage of complex media composition yeast extract. Further studies using chemically define medium, instead complex medium, could be a clue to the above question. Meanwhile, contrary to the sharp glucose consumption rate during the initial 20 h fermentation, the consumption rate of glycerol appears to be gradual slope; the intactness of Zwf on the pentose phosphate pathway during fed-batch fermentation will make extra flux to glyceraldehyde-3-P along with its precursors, thus causing catabolite repression of glycerol. A maximum DOI production of 38.0 g/L was reached at 50 h of fermentation, and then the DOI titer slightly decreased up to hour 60. Cell growth also seems to be in the stationary phase after 50 h fermentation, thus suggesting that there may be some relations between DOI production and cell stability in fed-batch fermentation. During the total 60 h of fermentation, the initial glucose level was set

to 30 g per liter of a culture medium. Considering that 10 g was additionally fed into the culture after 30 h fermentation, a total of 40 g of glucose as the main carbon source was converted to 38.0 g of DOI, meaning that a yield of approximately 95% was achieved. The yield of 95% seen in the present study is lower than the 99% yield achieved in a metabolically engineered recombinant strain of *E. coli* into which a natural DOI synthase–encoding *btrC* gene was introduced (Kogure et al., 2007), but the DOI titer was higher than what was reported in this previous publication (i.e., 38.0 vs. 29.5 g/L). Furthermore, considering the DOI productivity based on total fermentation time (i.e., 50 vs. 72 h), the DOI productivity obtained in this study was 0.76 g/(L.h) compared with 0.41 g/(L.h) in the previous study on the engineered *E. coli* recombinant. Our result represents ∼1.8-fold enhancement of DOI productivity. Meanwhile, when the titers obtained from shake-flask and fed-batch fermentations were compared, there was no significant difference (i.e., 37.2 vs. 38.0 g/L). But, considering with the time-dependent productivity (i.e., 60-h vs. 50-h), the DOI productivity during shake-flask fermentation [0.62 g/(L.h)] was meaningfully lower than fed-batch fermentation [0.76 g/(L.h)]. Consequently, it was confirmed that the expression of a codon-optimized DOI synthase–encoding $tobC_{opt}$ gene in a metabolically engineered cell factory of *B. subtilis*—in which both the Glc6P isomerase *pgi* gene and phosphoglucomutase *pgcA* gene (involved in the glycolytic metabolic pathway) are disrupted—led to an approximately 38 g/L DOI titer within 50 h of fermentation that employs glycerol (besides glucose) as an extra carbon and energy source for growth. Namely, mass production of the desired DOI could be attained via the fed-batch fermentation by the engineered cell factory constructed through convergence of metabolic engineering and synthetic biology.

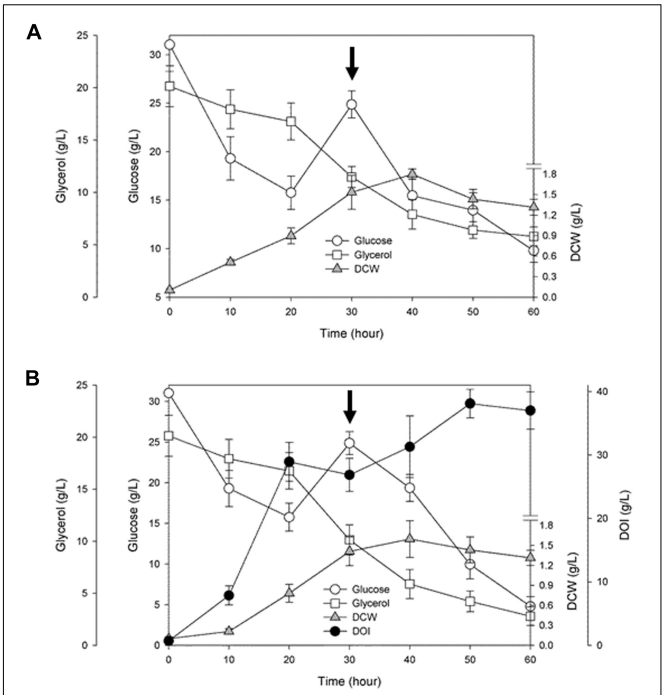

FIGURE 3 | Time courses of cell growth and 2-deoxy-*scyllo*-inosose (DOI) production, together with the profiles of glucose and glycerol consumed during fed-batch fermentation by the recombinant **(A)** BSDOI-11 (BSΔpgiΔpgcA), and **(B)** BSDOI-15 (BSΔpgiΔpgcA + *tobCopt*) strain. DCW represents dry cell weight, whereas the arrow shown at 30 h denotes when 10 g of glucose was added. Data were expressed as median (n = 4) ± standard deviations.

CONCLUSION

Here, we report that the titer, yield, and productivity of DOI obtained via fed-batch fermentation by newly engineered *B. subtilis* cell factories are at least equivalent to those in another study [on engineered *E. coli* recombinants] (Kogure et al., 2007). For the production of DOI via fermentation by the recombinants constructed herein, we employed a dual carbon source: glucose and glycerol. Besides, in the case of the above-mentioned study on engineered *E. coli* recombinants, glucose and mannitol (up to 4%, fed into the fermentation medium) were utilized for DOI production. Glycerol has been generated as the main byproduct of the manufacture of biodiesel, one of renewable energy sources for the substitution of petroleum, thus pointing to the potential utilization of glycerol as a carbon or energy source for industrial fermentation (da Silva et al., 2009). Hence, in comparison with cost-ineffective mannitol, glycerol is surely a favorable and cost-effective carbon source for industrial-scale fermentation. Although further optimization of the fermentation parameters (i.e., the ratio of carbon sources or the cell mass or density corresponding to the modification of carbon sources, and the time point of feeding) is required, it will be useful to determine whether other cost-effective and sustainable carbon sources can be utilized to reach an equally high titer or productivity by means of these engineered recombinants. In this study, for the expression of natural and artificial DOI synthase-encoding genes, a strong constitutive P_{43} promoter was employed as the control. However, the recent studies on promoter screening or its engineering for fine-tuned gene expression in *B. subtilis* host gave us a more details on the host expression patterns (Song et al., 2016; Liu et al., 2018), which are essential for synthetic biology and metabolic engineering approaches. Development of other inducible promoters and their application onto our host could be useful for industrial production of DOI. In addition, owing to the slight drop of the DOI titer after 50 h of fermentation, monitoring of DOI profiles during the fed-batch fermentation may be necessary for the maintenance of DOI stability. In particular, DOI synthase acts on Glc6P to biosynthesize DOI with the help of cofactor nicotinamide adenine dinucleotide (NAD) (Huang et al., 2005; Nango et al., 2008). Redox engineering has often been carried out and applied to various desired products (Qiao et al., 2017). Therefore, as a further study, redox engineering, or subsequent engineering of the NAD recycling (or regeneration) pathway in the *B. subtilis* recombinants constructed in this study, may be tested as a potential booster not only to enhance the DOI titer or productivity but also to develop cell factory platforms relevant to DOI-based pharmaceuticals and fine chemicals.

AUTHOR CONTRIBUTIONS

JP and YY conceived the project and wrote the manuscript. JHL, HH, NL, JWL, ES, HS, and HK designed and conducted all the experiments. JHL, HH, NL, YY, and JP analyzed the results.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02333/full#supplementary-material

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Single Amino Acid Substitution in Homogentisate Dioxygenase Affects Melanin Production in *Bacillus thuringiensis*

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Bacillus thuringiensis formulation losing its activity under field conditions due to UV radiation and photoprotection of *B. thuringiensis* based on melanin has attracted the attention of researchers for many years. Here, a single amino acid substitution (G272E) in homogentisate 1,2-dioxygenase was found to be responsible for pigment overproduction in *B. thuringiensis* BMB181, a derivative of BMB171. Disrupting the gene encoding homogentisate dioxygenase in BMB171 induced the accumulation of the homogentisic acid and provoked an increased pigment formation. To gain insights into homogentisate 1,2-dioxygenase in *B. thuringiensis*, we constructed a total of 14 mutations with a single amino acid substitution, and six of the mutant proteins were found to affect the melanin production when substituted by alanine. This study provides a new way to construct pigment-overproducing strains by impairing the homogentisate dioxygenase with a single mutation in *B. thuringiensis*, and the findings will facilitate a better understanding of this enzyme.

Keywords: *Bacillus thuringiensis*, tyrosine catabolism, homogentisate dioxygenase, site-directed mutagenesis, melanin

INTRODUCTION

Bacillus thuringiensis, a gram-positive spore-forming soil bacterium, has been widely used in biological pest control due to the formation of parasporal crystal proteins that are toxic to the larvae of various insect pests (Sudakin, 2003). However, the insecticidal activity of the crystal proteins would be reduced or destroyed under field conditions because of UV damage in sunlight (Pusztai et al., 1991). To solve this problem, researchers have proposed a series of strategies to protect insecticidal crystal proteins from UV damage (Sanchis et al., 1999; Manasherob et al., 2002; Jallouli et al., 2014), and one of them is the use of melanin, a photoprotective agent, to reduce the damaging effect of UV radiation on *B. thuringiensis* toxins (Liu et al., 1993; Ruan et al., 2002; Zhang et al., 2008; Sansinenea and Ortiz, 2015).

Melanins are polymers of phenolic and/or indolic compounds and classified into three main categories: eumelanins, pheomelanins, and allomelanins (Plonka and Grabacka, 2006). These black pigments are widely distributed in nature and can be found in species of all biological kingdoms, including humans, fungi, and bacteria (Plonka and Grabacka, 2006). Melanins provide free-living species a survival advantage in the environment by protecting against different exogenous stresses, such as UV-irradiation, reactive oxygen species (ROS), metals, and defensins (Nosanchuk and Casadevall, 2003, 2006; Heinekamp et al., 2012). Both eumelanins and pheomelanins are produced from the oxidation of tyrosine or phenylalanine to o-dihydroxyphenylalanine (DOPA) and dopaquinone via tyrosinases or laccases. Allomelanins include a heterogeneous group of polymers formed through the oxidation and polymerization of the intermediates such as dihydroxynaphthalene, homogentisic acid (HGA), γ-glutaminyl-4-hydroxybenzene, catechols, and 4-hydroxyphenylacetic acid (Plonka and Grabacka, 2006).

Homogentisic acid is derived from the tyrosine or phenylalanine catabolism pathway (one branch of tyrosine metabolism) and further oxidized to acetoacetic acid and fumaric acid (Turick et al., 2010). Pyomelanin is formed from the autoxidation and self-polymerization of HGA with the deactivation of homogentisate dioxygenase (HmgA) or the disruption of the gene encoding HGA-oxidase (Rodriguez-Rojas et al., 2009; Schmaler-Ripcke et al., 2009; Valeru et al., 2009). A deficiency of this enzyme in humans causes the metabolic disease alkaptonuria (AKU), leading to the excretion of HGA in the urine in a large amount and its deposition in different tissues (Millucci et al., 2012). The synthesis of pyomelanin has been investigated in a broad range of bacteria, such as *Aeromonas media, Burkholderia cepacia*, *Bacillus anthracis*, *Legionella pneumophila*, *Pseudoalteromonas*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Ralstonia solanacearum*, *Shewanella colwelliana*, and so on (Arias-Barrau et al., 2004; Valeru et al., 2009; Turick et al., 2010; Gonyar et al., 2015; Han et al., 2015; Wang et al., 2015; Ahmad et al., 2017; Zeng et al., 2017).

A number of studies have been performed to improve the photoprotection of the *B. thuringiensis* crystal proteins by producing melanin or increasing the melanin yield in *B. thuringiensis* cells. Some studies focused on screening *B. thuringiensis* mutants that can produce melanin in different conditions, and others attempted to construct recombinant *B. thuringiensis* strains with melanin production by genetic engineering (Hoti and Balaraman, 1993; Ruan et al., 2002; Saxena et al., 2002; Zhang et al., 2008; Sansinenea and Ortiz, 2015). It has been found that melanin could be produced by most *B. thuringiensis* strains in the presence of L-tyrosine at an elevated temperature (42°C), but the insecticidal Cry proteins could not be synthesized at this temperature (Ruan et al., 2004). In our previous work, a mutant, *B. thuringiensis* strain BMB181, was identified to be able to produce the brownish black pigment as an alternative melanin without tyrosine supplementation in the growth medium, and this strain could achieve a high melanin yield in different media without additional L-tyrosine (Liu et al., 2013). However, the mechanism for melanin production by BMB181 remains unclear. Here, the pigment produced by the strain BMB181 was found to be derived from homogentisate acid. The inactivation of HmgA by a G272E amino acid substitution resulted in pigmentation in the strain BMB181. Six single-point mutations in HmgA resulted in observable changes of melanin production in *B. thuringiensis*. This study offers valuable information about HmgA and provides a new way of constructing the pigment production strain of *B. thuringiensis*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are shown in **Table 1**. The *B. thuringiensis* strain BMB171 and its derivative BMB181 have been reported in previous studies (He et al., 2010; Liu et al., 2013). Bacteria were grown in Luria-Bertani (LB) medium at 37°C (*E. coli*) or 28°C (*B. thuringiensis*) and 200 rpm. The antibiotics, including kanamycin (50 μg/mL), erythromycin (25 μg/mL), and ampicillin (100 μg/mL), were added when necessary.

DNA Manipulation and Sequence Analysis

In this study, DNA was manipulated using the standard techniques as previously described (Green and Sambrook, 2012). The DNA of *B. thuringiensis* was extracted as previously reported (Andrup et al., 1993). The DNA fragments were amplified with the related primers, and the polymerase chain reaction (PCR) products were confirmed by DNA sequencing. The DNA sequences were analyzed using the DNASTAR software and the protein sequences were compared with those of other proteins using BLAST and CLUSTALX.

Transformation Techniques

Escherichia coli was transformed using $CaCl_2$-treated competent cells (Green and Sambrook, 2012), and *B. thuringiensis* was transformed by electroporation with the Bio-Rad Gene Pulser set (Bio-Rad, Hercules, CA, United States) (Peng et al., 2009).

High Performance Liquid Chromatography (HPLC) Analysis of Culture Filtrates

The HPLC analysis of culture filtrates was performed using an HPLC apparatus equipped with a variable wavelength UV-visible detector (CapLC 2487, Waters) and a C-18 end-capped column (10 μm, 4.6 mm × 150 mm; Elite). Briefly, the bacteria were grown in LB medium at 28°C and 200 rpm, and the culture supernatants were collected by centrifugation and filter sterilization. Next, 20 μL of the sample was injected into the column for HPLC. The mobile phase was 50 mM sodium phosphate buffer (pH 6.5)/methanol (80:20, v/v) at a flow rate of 0.5 mL/min as described by Fernandez-Canon and Penalva (1995). The chromatograms of standard solutions of HGA (from Sigma) were used as a reference to identify the corresponding HPLC peaks. The absorption maximum of HGA is 290 nm.

TABLE 1 | Bacterial strains and plasmids used in this study.

Strains or plasmids	Characteristics[a]	Origin or reference
Escherichia coli		
DH5α	*supE*44 Δ*lacU*169 (φ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Stored in this lab
Bacillus thuringiensis		
BMB171	A crystalliferous mutant of *B. thuringiensis*	He et al., 2010
BMB181	A mutant strain of BMB171, produce the brownish black pigment without tyrosine supplementation in the growth medium	Liu et al., 2013
BMB171Δ*hmgA*	BMB171 derivative, with a kanamycin insertion in *hmgA* gene	This work
BMB3141	derivative of BMB181, containing pBMB3141	This work
BMB3142	derivative of BMB181, containing pBMBL	This work
BMB3143	derivative of BMB171Δ*hmgA*, containing pHT304	This work
BMB3144	derivative of BMB171Δ*hmgA*, containing pBMB3144	This work
BMB3145	derivative of BMB171Δ*hmgA*, containing pBMB3145	This work
BMB3146a	derivative of BMB181, containing pBMB3144	This work
BMB3146b	derivative of BMB181, containing pBMB3145	This work
Plasmids		
pHT304	*E. coli* and *B. thuringiensis* shuttle vector; Amp^r, Erm^r	Arantes and Lereclus, 1991
pBMBL	Derivative of pHT304, containing a sporulation-dependent promoters BtI-BtII and the *cry1Ac* transcription terminator, Amp^r, Erm^r ≈7.6 kb	Stored in this lab, unpublished
pDG780	*E. coli* vector, Amp^r, Kan^r	Guerout-Fleury et al., 1995
pHT304-ts	Derivative of pHT304, containing a temperature-sensitive replicon in *B. thuringiensis*, ≈6.8 kb, Amp^r, Erm^r	Liu et al., 2010
pBMB3141	Derivative of pBMBL, containing the *hmgA* gene (1,173 bp) from BMB171, Amp^r, Erm^r	This work
pBMB3143	Derivative of pHT304-ts, a plasmid containing the *hmgA* gene fragments flanking the kanamycin resistance cassette Amp^r, Erm^r, Kan^r	This work
pBMB3144	Derivative of pHT304, carrying the *hmgA* gene operon from BMB171	This work
pBMB3145	Derivative of pHT304, carrying the *hmgA* gene operon from BMB181	This work
pBMB3146	Derivative of pHT304, containing the transcription promoter and terminator of *hmgA* gene operon, the gene of *hmgA* from BMB171	This work

[a] *Amp^r, ampicillin resistance; Erm^r, erythromycin resistance; Kan^r, kanamycin resistance.*

Isolation of the *hmgA* Gene

The *hmgA* gene (1,173 bp) encoding HmgA was amplified from *B. thuringiensis* BMB171 and BMB181 genomic DNA using the pair of primers, hmgA1 (5′-CGCGGATCCATGTTTTATCGTCACATGGGAG-3′, with the *Bam*HI recognition sequence underlined) and hmgA2 (5′-CGCGTCGACTTATTTCACAGTATATGAACCT-3′, with the *Sal*I recognition sequence underlined). The *hmgA* genes from BMB171 and BMB181 were designated as 171*hmgA* and 181*hmgA*, respectively.

Insertional Inactivation of the *hmgA* Gene

To verify the function of the *hmgA* gene, the gene disruption strain was constructed for BMB171 via homologous recombination using a temperature-sensitive shuttle vector pHT304-ts containing the temperature-sensitive replication origin (Liu et al., 2010). Briefly, a 503-bp fragment and a 600-bp fragment, corresponding to the DNA regions upstream and downstream of the open reading frame of the *hmgA* gene in the strain BMB171, were generated by PCR using the primer pairs HmgA-up-1 (5′-CGCGGATCCTGGGAGAACTACCTCATAAAC-3′)/HmgA-up-2(5′-CCGGAATTCGCTATTCGCCTCTACAACA-3′) and HmgA-down-1(5′-CCGGTCGACAATTGTTAGAGCATAGTCCG-3′)/HmgA-down-2(5′-CGGGGTACCATGAACCTTGTTCAATCCAG-3′) and digested with *Bam*HI- *Eco*RI and *Acc*I-*Kpn*I, respectively. A kanamycin resistance cassette (1,514 bp) was acquired by digesting plasmid pDG780 (Guerout-Fleury et al., 1995) with *Eco*RI-*Acc*I. These three fragments were cloned into the plasmid pHT304-ts at the *Bam*HI-*Kpn*I site. The resulting plasmid, pBMB3143, was transformed into the strain BMB171 by electroporation.

The mutants were selected as follows. Specifically, the transformants were cultured in LB medium with kanamycin (50 μg/mL) for 8 h, followed by incubation at 42°C for 4 days to eliminate unintegrated temperature-sensitive plasmids. Finally, the mutant strains that were resistant to kanamycin but sensitive to erythromycin colonies were confirmed by PCR using appropriate primers and sequencing.

Genetic Complementation Analysis

For genetic complementation analysis, complementation plasmids were prepared by using the shuttle vector pHT304 (Arantes and Lereclus, 1991) and its derivative pBMBL (unpublished data) that contained a sporulation-dependent promoter BtI-BtII and the *cry1Ac* transcription terminator. The amplified fragment *171hmgA* was cloned into the vector pBMBL to yield the plasmid pBMB3141. To construct the plasmids pBMB3144 (carrying 171*hmgA*

operon) and pBMB3145 (carrying 181*hmgA* operon), the *hmgA* gene operon region (about 3.9 kb) containing the genes encoding 4-hydroxyphenylpyruvate dioxygenase (HppD), HmgA, and fumarylacetoacetate hydrolase (FahA) was amplified, respectively, from the strains BMB171 and BMB181 using special primers HMGAop-S (5′-CGCGGATCCAGATATATAAATACAATCATTC-3′, with the *Bam*HI recognition sequence underlined) and HMGAop-A (5′-CGCGTCGACTCTTTCACTCCTCCAAGTTT-3′, with the *Sal*I recognition sequence underlined) and subsequently cloned into pHT304. Finally, the two recombinant plasmids were transformed into the pigmented *B. thuringiensis* strains by electroporation separately.

Measurement of the Bacterial Growth Curve and the Pyomelanin Production

The growth curve and the pigment production of the *B. thuringiensis* strains and their derivatives were evaluated according to optical density (OD). To monitor the growth curves of the strains, the bacteria were inoculated to 100 mL of LB medium (the flask volume is 500 mL) and incubated under shaking at 28°C and 200 rpm, followed by the OD measurement of the cultures at 600 nm (OD_{600}) at different time intervals. The melanin production of the strains was quantified by testing the absorbance of the centrifuged culture supernatant at 400 nm (OD_{400}) at the indicated time points (Ruan et al., 2002; Liu et al., 2013).

Alanine Scanning Site-Directed Mutagenesis

For performing alanine scanning site-directed mutagenesis, we constructed the plasmid pBMB3146 (a derivative of pHT304, containing the promoter region and the terminator region of the *hmgA* gene operon and the 171*hmgA* gene). Briefly, the promoter region of the *hmgA* gene operon (394 bp) was amplified from the strain BMB171 genomic DNA using the pair of primers, HMGAop-S (5′-CGCGGATCCAGATATATAAATACA ATCATTC-3′, with the *Bam*HI recognition sequence under lined) and HMGAop-A2 (5′-CTCCCATGTGACGATAAAAC ATAATATCTTCATCTCCCTGTAA-3′). Next, the 1,405-bp PCR product, containing the 171*hmgA* gene and the terminator region of the *hmgA* gene operon, was amplified using primers HMGA-S2 (5′-TTACAGGGAGATGAAGATATTATGTTTTAT CGTCACATGGGAG-3′) and HMGAop-A (5′-CGCGTCGAC TCTTTCACTCCTCCAAGTTT-3′, with the *Sal*I recognition sequence underlined). Finally, the vector pBMB3146 was generated by connecting the two fragments with overlapping PCR (Higuchi et al., 1988) using primers HMGAop-S and HMGAop-A, digesting the overlapping PCR fragment with *Bam*HI and *Sal*I, and inserting it into the shuttle vector pHT304.

A total of fourteen amino acid residues of HmgA were mutated to alanine residues by overlapping PCR using the primers shown in **Table 2**, with vector pBMB3146 as the template. The PCR fragments were cloned separately into the vector pHT304 between the *Bam*HI and *Sal*I restriction sites. The recombinant plasmids were transformed into *E. coli* DH5a and the positive clones were screened by restriction enzyme analysis.

TABLE 2 | Primers used for site-directed mutagenesis.

Primers	Oligonucleotides (5′→3′)[a]	Use
HMGAop-S	CGCGGATCCAGATATATAAATACAATCATTC	
HMGAop-A	CGCGTCGACTCTTTCACTCCTCCAAGTTT	
Hm89-R	GTGATGCAATAAGT**GCA**CGGAATTTCATACT	G89A substitution
Hm89-L	AGTATGAAATTCCG**TGC**ACTTATTGCATCAC	
Hm116-R	ATTATTTCTATCGT**GCA**GGTGATGGCGACGA	N116A substitution
Hm116-L	TCGTCGCCATCACC**TGC**ACGATAGAAATAAT	
Hm119-R	ATCGTAATGGTGAT**GCA**GACGAAATGTTATT	G119A substitution
Hm119-L	AATAACATTTCGTC**TGC**ATCACCATTACGAT	
Hm120-R	GTAATGGTGATGGC**GCA**GAAATGTTATTTGT	D120A substitution
Hm120-L	ACAAATAACATTTC**TGC**GCCATCACCATTAC	
Hm128-R	TATTTGTTCATTAT**GCA**ACAGGGAAAATTGA	G128A substitution
Hm128-L	TCAATTTTCCCTGT**TGC**ATAATGAACAAATA	
Hm136-R	AAATTGAAACGATG**GCA**GGAACGATTCACTA	F136A substitution
Hm136-L	TAGTGAATCGTTCC**TGC**CATCGTTTCAATTT	
Hm219-R	TTGTCGTAATGACA**GCA**TCAAGAGGCTATAT	K219A substitution
Hm219-L	ATATAGCCTCTTGA**TGC**TGTCATTACGACAA	
Hm241-R	TTGTGGGATGGGAT**GCA**TATTTATATCCGTG	G241A substitution
Hm241-L	CACGGATATAAATA**TGC**ATCCCATCCCACAA	
Hm245-R	ATGGCTATTTATAT**GCA**TGGGTATTTAATGT	P245A substitution
Hm245-L	ACATTAAATACCCA**TGC**ATATAAATAGCCAT	
Hm261-R	TTACAGGGCGCATT**GCA**CAGCCACCGCCAGT	H261A substitution
Hm261-L	ACTGGCGGTGGCTG**TGC**AATGCGCCCTGTAA	
Hm300-R	CATATTATCATAGT**GCA**GTTAATAGTGATGA	N300A substitution
Hm300-L	TCATCACTATTAAC**TGC**ACTATGATAATATG	
Hm323-R	AAGGTGTGGAAGAA**GCA**TCTATTACACTTCA	G323A substitution
Hm323-L	TGAAGTGTAATAGA**TGC**TTCTTCCACACCTT	
Hm334-R	CGAGCGGGATTCCC**GCA**GGACCGCATCCGGG	H334A substitution
Hm334-L	CCCGGATGCGGTCC**TGC**GGGAATCCCGCTCG	
Hm336-R	GGATTCCCCATGGA**GCA**CATCCGGGGAAAAC	P336A substitution
Hm336-L	GTTTTCCCCGGATG**TGC**TCCATGGGGAATCC	

[a] *The restriction sites included in the oligonucleotide sequences are underlined. Nucleotide codons encoding the mutated amino acids are highlighted in bold types.*

Before transformation into the pigmented *B. thuringiensis* strain BMB171Δ*hmgA*, all the resulting mutant plasmids were sequenced to ensure that the proper mutations were maintained.

Sequence Accession Number

The sequences reported in this paper have been submitted to the GenBank. The accession number for the complete genome of the strain BMB171 is CP001903.1. The accession number for the HmgA (locus tag is BMB171_C0216) is ADH05034.1.

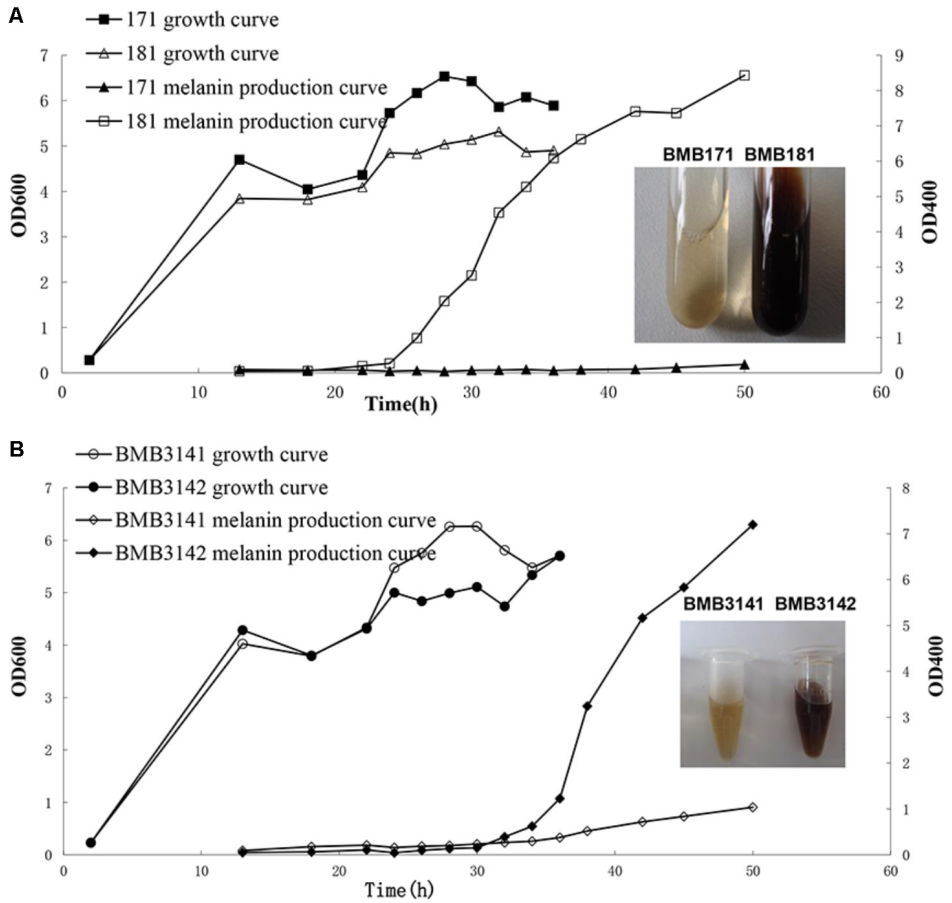

FIGURE 1 | Growth and melanin production curves of *B. thuringiensis* strains in LB medium. **(A)** Growth and melanin production curves of BMB171 and BMB181 in LB medium. **(B)** Growth and melanin production curves of BMB3141 and BMB3142 in LB medium. BMB3141, a derivative of BMB181, contained pBMB3141 that harbors 171*hmgA*; BMB3142, a derivative of BMB181, contained pBMBL, as a negative control.

RESULTS

Pigment Results From Polymerization of Homogentisate

BMB181, a *B. thuringiensis* mutant with high melanin production, was obtained after subculturing the strain BMB171 for several generations at 42°C (Liu et al., 2013). The red pigment produced by the strain BMB181 turned dark brown with the extension of incubation time (**Figure 1A**). Pigments can be formed from the oxidation and polymerization of compounds such as DOPA and HGA (Plonka and Grabacka, 2006). In our early work, we found that the pigment produced by the strain BMB181 has nothing to do with DOPA (data not shown). Therefore, we test whether HGA is the precursor of pigment produced by the strain BMB181. Here, ascorbic acid was added as an antioxidant to prevent HGA from oxidation. No pigment was observed when ascorbic acid (2 mM) was added to the cultures of the strain BMB181 (data not shown). Strains BMB171 and BMB181 were cultured in LB liquid media under shaking at 28°C for 24 h and the culture samples were taken for HPLC analysis after centrifugation and filtration, using the commercially available HGA as the standard. A peak corresponding to HGA (with a retention time of 8.68 min) was identified in the culture supernatants of the strain BMB171 with HGA added to the culture during the logarithmic growth phase (**Figures 2A,B**). The peak with the same chromatography retention time as HGA was identified in the culture supernatants of the strain BMB181 (**Figure 2C**), suggesting that homogentisate could be secreted by the pigmented strain BMB181 and the pigment produced by the strain probably resulted from the accumulation and polymerization of homogentisate.

Identification of Amino Acid Substitution in HmgA

Considering that the melanin production from homogentisate induced by a deficiency of HmgA in organisms is associated with the tyrosine metabolism pathway (**Figure 3A**), we analyzed the genome of BMB171 and found that BMB171 carried the genes encoding HppD, HmgA, and FahA (**Figure 3B**). The relationship between the biosynthesis of the pigment in the strain BMB181 and HmgA was tested by amplifying the *hmgA* gene from the BMB181 genomic DNA and sequencing. After aligning

the inferred amino acid sequence of HmgA from the publicly available BMB171 sequences (He et al., 2010), a glycine was found to be replaced by a glutamate at residue 272 in HmgA in the pigmented strain BMB181.

Whether this amino acid substitution is responsible for the observed pigmented phenotype was tested by performing a complementation analysis. Specifically, the plasmid pBMB3141 containing the 171*hmgA* gene was transformed into BMB181 to create the transformant strain BMB3141 (171$hmgA^+$), and BMB3142, a BMB181 derivative containing the plasmid pBMBL was used as a control. It was found that BMB3141 (171$hmgA^+$) was reverted to a non-pigmented phenotype, with no significant increase observed in the OD_{400} of the supernatant relative to the control (**Figure 1B**). These results indicate that the G272E version of HmgA is responsible for melanin production in the *B. thuringiensis* pigmented strain BMB181.

Disruption of HmgA Results in Pigment Formation Due to Inactivation of Homogentisate Dioxygenase

To verify that the lack of a functional HmgA is responsible for the pigment formation of *B. thuringiensis*, a *hmgA* insertion mutant strain was constructed by homologous recombination. Specifically, the plasmid pBMB3143 containing *hmgA* gene fragments flanking the kanamycin resistance cassette was constructed and transferred into the strain BMB171. The recombinants with double-crossover homologous recombination integration in the resident *hmgA* gene were selected and verified by PCR. The strain carrying the *hmgA*::*kan* disruption, named BMB171Δ*hmgA*, was able to produce the brownish black pigment in cultures (**Figure 4A**). HPLC analysis showed that HGA was secreted and accumulated in the culture supernatants of BMB171Δ*hmgA* (**Figure 2D**).

The complete *hmgA* gene operons from the strains BMB171 and BMB181 were amplified and used for the complementation analysis, and the plasmids containing different alleles of *hmgA* were named as pBMB3144 (carrying the operon containing the *hmgA* gene from BMB171) and pBMB3145 (carrying the operon containing the *hmgA* gene from BMB181). The plasmids expressing either allele of *hmgA* were transferred into the strain BMB171Δ*hmgA* (pigmented phenotype). It was found that the transformant harboring the 171*hmgA* gene operon (strain BMB3144) exhibited a non-pigmented phenotype, while the transformant harboring the 181*hmgA* variant operon (strain BMB3145) remained pigmented (**Figure 4B**). The same result was found when the two plasmids were introduced into the strain BMB181 (**Figure 4C**). These data suggest that the HmgA variant from the BMB181 mutant with residue Gly272 replaced by the residue Glu is not functional and ultimately results in pigment overproduction, while the functional HmgA enzyme from the strain BMB171 results in the absence of pigment from HGA.

Mutational Analysis of HmgA in *B. thuringiensis*

To further test whether other single residue mutations have the same effect as G272E on melanin production, we designed 14 single-point mutations (G89A, N116A, G119A, D120A, G128A, F136A, K219A, G241A, P245A, H261A, N300A, G323A, H334A, and P336A) in 171HmgA based on the sequence alignment and the secondary structure of 171HmgA (**Figure 5**). A *trans*-complementation test was performed by introducing all the sequences under the control of the shuttle vector pHT304 separately into the pigmented strain BMB171Δ*hmgA*. Among the fourteen transformants, eight (containing G89A, N116A, G119A, D120A, K219A, P245A, N300A, and G323A, respectively) showed a non-pigmented phenotype, while the other six (containing G128A, F136A, G241A, H261A, H334A, and P336A, respectively) showed a pigmented phenotype (**Table 3**), implying that these six amino acid substitutions could result in the function loss of HmgA, making it unable to restore the non-pigment phenotype.

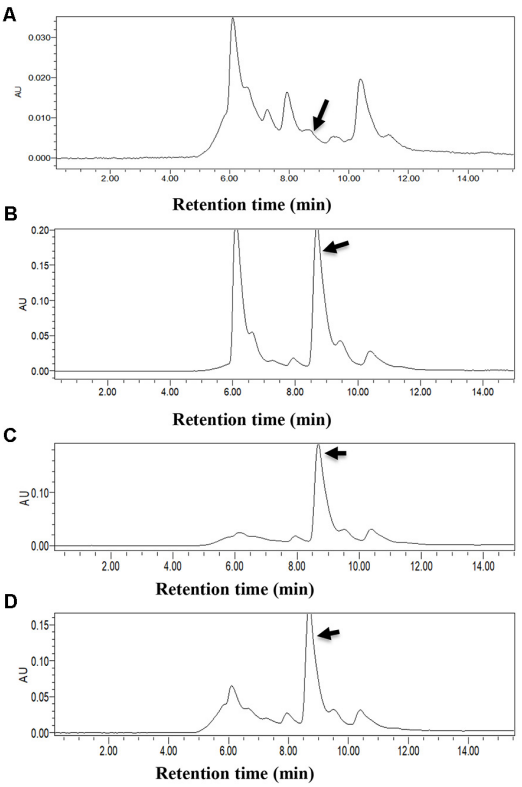

FIGURE 2 | HPLC analysis of culture filtrates of BMB171, BMB181, and BMB171Δ*hmgA*. Homogentisate acid was identified by HPLC and is indicated by black arrows. **(A)** Supernatants of BMB171 (with ascorbic acid), as a negative control. **(B)** Supernatants of BMB171 (with ascorbic acid and Homogentisate), as positive control. **(C)** Supernatants of BMB181 (with ascorbic acid). **(D)** Supernatants of BMB171Δ*hmgA* (with ascorbic acid).

DISCUSSION

In aerobic organisms, L-Tyrosine degradation via homogentisate (HGA) is initiated by the conversion of tyrosine to 4-hydroxyphenylpyruvate by tyrosine aminotransferases, followed by the formation of HGA from 4-hydroxyphenylpyruvate

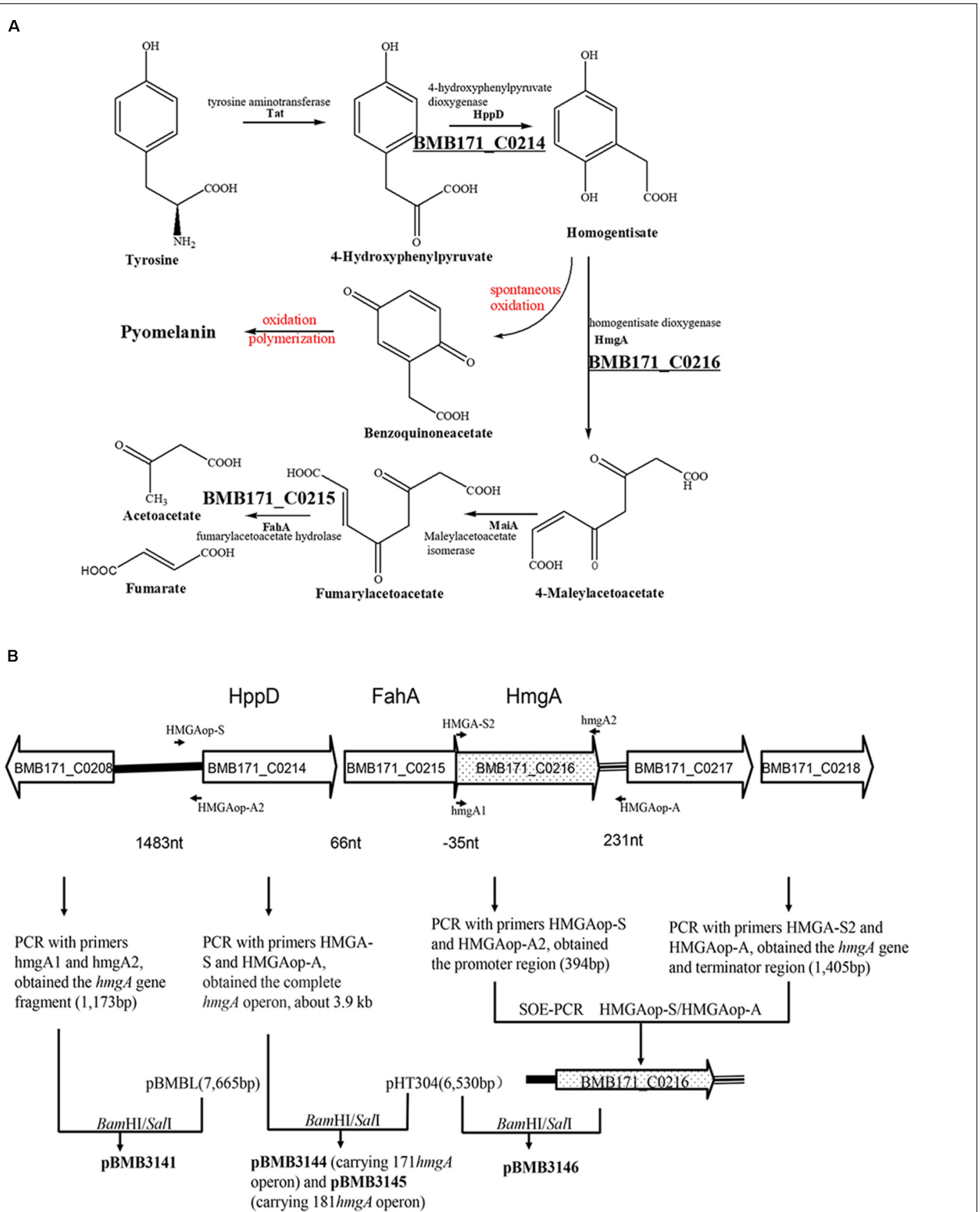

FIGURE 3 | Pathway for the catabolism of homogentisate. **(A)** L-Tyrosine metabolism pathway via homogentisate [modified from reference (Schmaler-Ripcke et al., 2009)]. **(B)** Organization of the genes putatively involved in the catabolism of homogentisate in *B. thuringiensis* BMB171 and scheme for the construction of plasmid vectors. The black arrows indicate the primers used here. Enzymes encoded by the respective genetic loci in *B. thuringiensis* BMB171 are BMB171_C0208, HAD superfamily hydrolase; BMB171_C0214, HppD, 4-hydroxyphenylpyruvate dioxygenase; BMB171_C0215, FahA, fumarylacetoacetate hydrolase; BMB171_C0216,

(Continued)

FIGURE 3 | Continued
HmgA, homogentisate 1,2-dioxygenase; BMB171_C0217, amino acid permease; BMB171_C0218, MFS transporter. The vector pBMB3141, a derivative of pBMBL, contained the *hmgA* gene (1,173 bp) amplified from the genomic DNA of BMB171 by using primers hmgA1 and hmgA2. The vector pBMB3144 carried the *hmgA* gene operon from BMB171, and the vector pBMB3145 carried the *hmgA* gene operon from BMB181. The *hmgA* gene operon region (about 3.9 kb) was amplified by using primers HMGAop-S and HMGAop-A. The vector pBMB3146, a derivative of pHT304, contained the transcription promoter region and the terminator region of the *hmgA* gene operon and the *hmgA* gene from BMB171. The promoter region (394 bp) was amplified from the genomic DNA of BMB171 by using primers HMGAop-S and HMGAop-A2. The *hmgA* gene (1,173 bp) and the terminator region (232 bp) of the *hmgA* gene operon was amplified from the genomic DNA of BMB171 by using primers HMGA-S2 and HMGAop-A.

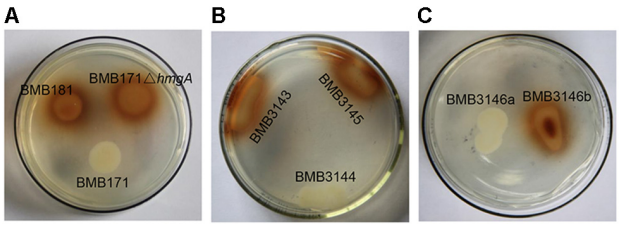

FIGURE 4 | Phenotypes of *B. thuringiensis* strains grown on LB agar plates. **(A)** Phenotypes of BMB171, BMB181, and BMB171Δ*hmgA*. **(B)** Phenotypes of BMB3143, BMB3144, and BMB3145. BMB3143, a derivative of BMB171Δ*hmgA*, contained pHT304, as a negative control; BMB3144, a derivative of BMB171Δ*hmgA*, contained pBMB3144 harboring the *hmgA* gene operon from BMB171; and BMB3145, a derivative of BMB171Δ*hmgA*, contained pBMB3145 harboring the *hmgA* gene operon from BMB181. **(C)** Phenotypes of BMB3146a and BMB3146b. BMB3146a, a derivative of BMB181, contained pBMB3144; BMB3146b, a derivative of BMB181, contained pBMB3145.

by HppD and the oxidation of HGA to maleylacetoacetate (MAA) by HmgA. MAA is isomerized by maleylacetoacetate isomerase (MaiA) to fumarylacetoacetate, which is subsequently hydrolyzed by FahA to fumarate and acetoacetate (Schmaler-Ripcke et al., 2009; Valeru et al., 2009). The genes encoding these enzymes (HppD, HmgA, FahA, and MaiA) are adjacent on the chromosome of several organisms such as *Aspergillus fumigatus, Pseudomonas chlororaphis*, and *Vibrio cholerae* (Kang et al., 2008; Schmaler-Ripcke et al., 2009; Valeru et al., 2009). In the KEGG pathway database, three genes (BMB171_C1377, BMB171_C1351, and BMB171_C2647) are predicted to encode the aminotransferases involved in the conversion of L-tyrosine to 4-hydroxyphenylpyruvate. The three enzymes (HppD, HmgA, and FahA) in *B. thuringiensis* BMB171 are probably transcribed as part of an operon as indicated by the genomic analysis (**Figure 3B**). Analyzing the genome sequences of *B. thuringiensis* strains of different H serotypes, we found that the gene operon for L-Tyrosine degradation via HGA is located on every genome (data not shown). However, the genes responsible for the isomerization of MAA cannot be directly identified in *B. thuringiensis* by analyzing the genomic data. This implies that the genes responsible for the MAA metabolism of *B. thuringiensis* might be different from that of the reported genes. Even so, we found that the inactivation of HmgA leads to pyomelanin hyperproduction in *B. thuringiensis* strains. We speculate that *B. thuringiensis* strains have the potential to produce pyomelanin via HGA through the tyrosine metabolism pathway by inactivating HmgA.

HmgA is involved in the catabolism of the phenylalanine and tyrosine pathway. The structure of the human HmgA shows that the enzyme forms a hexamer arrangement consisting of a dimer of trimers (Titus et al., 2000). HmgA has been predicted to belong to the cupin-like superfamily[1], which refers to a β-barrel structural domain, on the basis of the primary sequence. Two conserved histidine-containing motifs provide the signature sequence for the cupin superfamily (Dunwell et al., 2001). Despite the considerable variability of the HmgA proteins from different organisms in their primary amino acid sequences (from 22 to 65%), the residues involved in coordinating Fe^{2+} are highly conserved in the HmgA proteins from bacteria, fungi, plants, invertebrates, and humans (**Figure 5**). Three residues, including two residues His298 and Glu305 in motif 1, and one residue His334 in motif 2, are responsible for the metal ion binding of the 171HmgA protein (**Figure 5**). Additionally, the secondary structure of 171HmgA is composed of many β-strands (**Figure 5**). In this study, we found that a G272E mutation in HmgA resulted in pigment overproduction in the *B. thuringiensis* strain BMB181. Residues 272–274 of HmgA form a loop structure connecting two β-strands (β19 and β20). Gly272 is highly conserved in different HmgAs and the equivalent in the human HmgA is Gly309 (**Figure 5**). A G309V missense mutation has previously been found in AKU patients (Nemethova et al., 2016). These findings suggest that the residue Gly272 plays a very important role in the enzyme activity. To verify whether other residues in the loops or adjacent to the loops have the same effect as the residue Gly272, we designed 14 single-point mutants. Nine of the residues (Gly89, Gly119, Gly128, Phe136, Pro245, His261, Asn300, Gly323, and Pro336) are located in different loop regions, and the other five (Asn116, Asp120, Lys219, Gly241, and His334) are located at the start or end of different β-strands adjacent to the loop-forming residues. The result shows that only six mutants (G128A, F136A, G241A, H261A, H334A, and P336A) have an effect on melanin production (**Table 3**). The activity of the enzyme can be disrupted by mutations in many different ways. Some will directly or indirectly affect the active site, others will interfere with the folding of the subunit, and some will affect intersubunit interactions (Rodriguez et al., 2000). The relationship between these residues and the catalytic activity needs to be further confirmed by biochemical characterization and crystal structure.

Previous studies have shown that residues in many different sites of homogentisate 1,2 dioxygenase from humans are essential for enzyme function (Rodriguez et al., 2000; Vilboux et al., 2009; Zatkova, 2011). For instance, single amino acid

[1]https://www.ncbi.nlm.nih.gov/cdd/

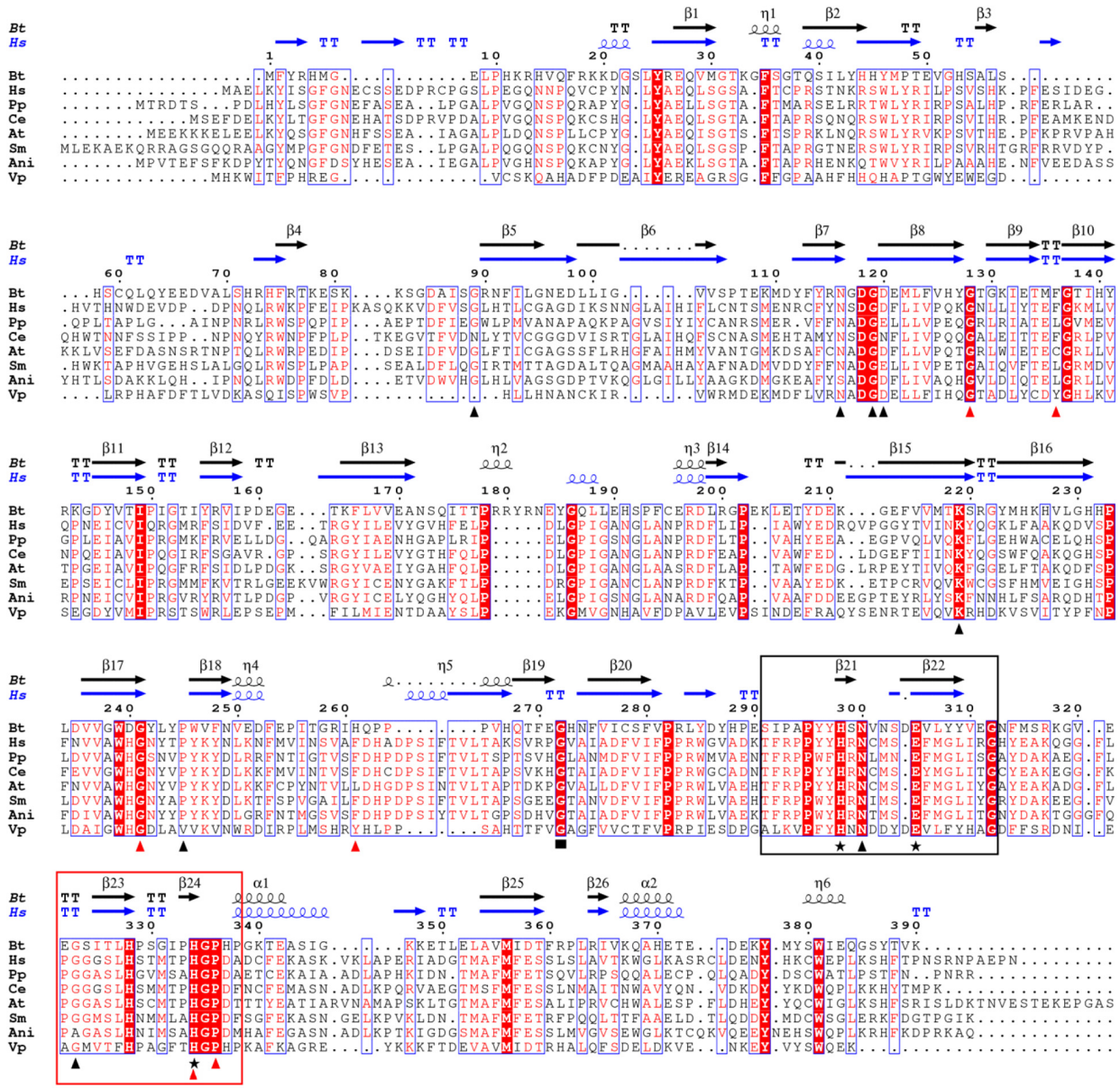

FIGURE 5 | Sequence alignment of HmgA proteins from different organisms. The positions of the two conserved cupin motifs are boxed. Motif 1 and motif 2 are shown in the black box and red box, respectively. Amino acid substitutions in 171HmgA are marked by triangles. The red triangles point to the residues of 171HmgA that when replaced by alanine residues affect pigment production. The black triangles indicate that the residues of 171HmgA, when mutated to alanine had no effect on pigment production. The black stars indicate the conserved residues responsible for iron binding. The black rectangle shows the amino acid substitution (G272E) in BMB181. *B. thuringiensis*, homogentisate 1,2-dioxygenase from *B. thuringiensis* BMB171 (ADH05034.1); Hs, homogentisate 1,2-dioxygenase from *Homo sapiens* (CAA99340.1); Pp, homogentisate 1,2-dioxygenase from *Pseudomonas putida* (AAO12527.1); Ce, 2,5 dihydroxyphenylacetate oxidase from *Caenorhabditis elegans* (AAD00776.1); At, homogentisate 1,2-dioxygenase from *Arabidopsis thaliana* (AAD00360.1); Sm, homogentisate dioxygenase from *Sinorhizobium meliloti* (AAD29874.1); Ani, 2,5 dihydroxyphenylacetate oxidase from *Aspergillus nidulans* (AAC49071.1); and Vp, homogentisate 1,2-dioxygenase from *Vibrio parahaemolyticus* 10329 (EGF43769.1). The sequences were aligned with ClustalW (Chenna et al., 2003) and ESPript (Robert and Gouet, 2014).

substitutions at different positions of HmgA can result in pyomelanin overproduction in AKU patients (Rodriguez et al., 2000; Vilboux et al., 2009; Zatkova, 2011). The deduced amino acid sequence of 171HmgA consists of 390 amino acids and shares 24% identity with the HmgA from humans. The single-residue substitution in the 13 equivalents to the aforementioned 14 mutational residues in the human HmgA sequence would result in the loss of enzyme activity and pigment production (**Table 3**; Zatkova, 2011; Usher et al., 2015). These results indicate that five residues (G128, F136, G241, H334, and P336) are more conservative than the others (G89, N116, G119, D120, K219, P245, N300, and G323) in HmgA, and

TABLE 3 | Site-directed mutagenesis in HmgA.

Transformants of BMB171 (Δ*hmgA*)	Mutation position	Amino acid change	Phenotype[a]	Equivalent residue(s) in human HmgA	Amino acid changes in AKU patients (Zatkova, 2011; Usher et al., 2015)[b]
BMB3147	hmgA89	G89A	N	115G	G115R
BMB3148	hmgA116	N116A	N	149N	N149K
BMB3149	hmgA119	G119A	N	152G	G152A
BMB3150	hmgA120	D120A	N	153D	D153G
BMB3151	hmgA128	G128A	P	161G	G161R
BMB3152	hmgA136	F136A	P	169F	F169L
BMB3153	hmgA219	K219A	N	248K	K248E
BMB3154	hmgA241	G241A	P	270G	G270R
BMB3155	hmgA245	P245A	N	274P	P274L
BMB3156	hmgA261	H261A	P	290F	?
BMB3157	hmgA300	N300A	N	337N	N337D
BMB3158	hmgA323	G323A	N	360G	G360R/A
BMB3159	hmgA334	H334A	P	371H	H371R
BMB3160	hmgA336	P336A	P	373P	P373L

[a] *N: no pigment; P: pigment.*
[b] *?: no single amino acid substitution was found in the residue F290 in AKU patients in the present study.*

mutations in residues G128, F136, G241, H334, and P336 could affect the production of pyomelanin in both humans and *B. thuringiensis*.

Pyomelanin production has been studied in different bacteria. The accumulation of pyomelanin does not affect the growth characteristics nor the expression of key virulence factors of *B. anthracis* (Han et al., 2015). Notably, the mutant *P. aeruginosa* strain PA14Δ*hmgA* is significantly more virulent than the wild-type PA14 as PA14Δ*hmgA* can kill the nematodes at a greatly accelerated rate compared with the wild-type PA14 (Harmer et al., 2015). Additionally, pyomelanin has been identified as the primary melanin produced by the *A. media* strain WS through the autoxidation and self-polymerization of HGA (Wang et al., 2015). The melanin produced by the strain WS serves as an excellent photoprotective agent for BTI against UV and sunlight radiation (Wan et al., 2007). A previous study has shown that the pigment produced by the *B. thuringiensis* strain BMB181 protects against UV radiation (Liu et al., 2013). To test the possible role of pyomelanin in H_2O_2 resistance, we estimated the viability of the culture of the strain BMB171 under H_2O_2 treatment and found that the supernatant from the mutant culture was able to protect the vegetative cells from the effect of H_2O_2 based on the growth curves (data not shown). We speculate that the pyomelanin produced by *B. thuringiensis* strains may have a significant synergistic effect on the crystal proteins against nematodes and other insect pests by not affecting the growth and the expression of key virulence factors of *B. thuringiensis* and protecting *B. thuringiensis* cells from the stresses such as H_2O_2 and UV during the life cycle.

In this study, we constructed several plasmid vectors for complementation analysis (**Figure 3B**). The plasmid pBMB3141 was constructed using the vector pBMBL (unpublished data) and the single *hmgA* gene fragment for the complementation analysis. However, we found that the strain BMB181 could not completely restore the non-pigment phenotype with the plasmid pBMB3141 transformed into it, and the pigment appeared after culturing the transformant BMB3141 (a derivative of BMB181 containing pBMB3141) in LB medium for over 40 h (**Figure 1B**), suggesting that the promoter of the vector pBMBL, a sporulation-dependent promoter (BtI-BtII), is not suitable for this restoration test. To solve this problem, we tried to construct a plasmid vector that contained its own promoter and terminator region of the *hmgA* gene for complementation analysis. As shown in **Figure 3B**, the genes *hppD*, *fahA*, and *hmgA* are organized as an operon as indicated by the genomic analysis, and the transcription of the operon would be influenced with the *hmgA* gene being interrupted by the kanamycin resistance cassette in the strain BMB171Δ*hmgA*. Thus, we constructed the plasmid vectors (**Figure 3B**) that contained the complete *hmgA* gene operon from strains BMB171 and BMB181 separately for the complementation analysis (**Figures 4B,C**). The functions of HmgA variants in melanin production were also tested by the complementation analysis. We firstly constructed the vector pBMB3146 that contained the promoter and terminator regions of the *hmgA* gene operon and the *hmgA* gene (**Figure 3B**). All of the point mutations in HmgA were carried out by gene splicing by overlap extension PCR (SOE PCR) with pBMB3146 as the template. By introducing the plasmids containing different *hmgA* gene variants into the pigmented strain BMB171Δ*hmgA*, the roles of different HmgA variants was preliminarily determined by the phenotypes (pigmented or non-pigmented) (**Table 3**). This indicates that some of these residues play important roles in enzyme activity. Nevertheless, the exact roles of these residues for the enzyme HmgA could not be

determined based only on the aforementioned data. The difference between HmgA variants should be distinguished by catalytic mechanisms in further related research.

In summary, *B. thuringiensis* strains could produce pyomelanin via HGA upon the deactivation of the HmgA or the disruption of the *hmgA* gene. We found that a G272E amino acid substitution in HmgA resulted in pigmentation. Several other residues in the loops or adjacent to the loops have the same effect as the residue Gly272 on the enzyme activity and melanin production. It is possible to generate more mutations in the *hmgA* gene and reintroduce them into *B. thuringiensis* and in many cases obtain a mutant phenotype (pigment overproducer). This approach has potential use to producing *B. thuringiensis* strains more resistant to UV. This is also an interesting model to study the human gene responsible for alkaptonuria.

AUTHOR CONTRIBUTIONS

WY, LR, and MS designed the research. WY and JT performed the research. WY, JZ, LR, DP, and MS analyzed the data. WY, LR, and MS wrote the manuscript.

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Microbial Platform for Terpenoid Production: *Escherichia coli* and Yeast

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Terpenoids, also called isoprenoids, are a large and highly diverse family of natural products with important medical and industrial properties. However, a limited production of terpenoids from natural resources constrains their use of either bulk commodity products or high valuable products. Microbial production of terpenoids from *Escherichia coli* and yeasts provides a promising alternative owing to available genetic tools in pathway engineering and genome editing, and a comprehensive understanding of their metabolisms. This review summarizes recent progresses in engineering of industrial model strains, *E. coli* and yeasts, for terpenoids production. With advances of synthetic biology and systems biology, both strains are expected to present the great potential as a platform of terpenoid synthesis.

Keywords: terpenoid, *Escherichia coli*, yeast, synthetic biology, MEP pathway, MVA pathway, strain engineering

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INTRODUCTION

Terpenoids comprise a vast family of the most abundant (>55, 000 known members) natural products with diverse biological functions including cell integrity, hormones, electron transport, and photosynthetic machinery. The extracted products from some herbs or animal liver are realized as flavors, fragrances, colorants, commodity chemicals, vitamins, and pharmaceuticals from ancient time. Nevertheless, their low yields from natural sources limit mass production of terpenoid for industrial application. For instance, *Artemisia annua* (Qinghao) just yields artemisinin of <0.8% by dry biomass weight (Zyad et al., 2018), which severely restricts commercialization of this antimalarial drug. Humans have employed microbes to produce beverages (e.g., *Saccharomyces cerevisiae*) before civilization, and antibiotics (e.g., *Penicillium chrysogenum*) at an industrial scale since the Second World War (Nielsen and Keasling, 2016). Nowadays, advances in metabolic engineering enable us to build microbial cell factories to generate many interesting products, of which *Escherichia coli* and yeast are the well-characterized hosts for efficient and large-scale production. Since Amyris Inc., announced a record low manufacturing cost of $1.75 per liter for farnesene from yeast in 2015, microbial farnesene has attracted a lot of interests from industry[1].

[1] http://investors.amyris.com/press-releases

This review describes progress in biosynthesis of the diverse terpenoids in *E. coli* and yeast, where fantastic technologies are harnessed for development of microbial cell factories. It illuminated the great potential of *E. coli* and yeast on tackling a complexity of biosynthesis of the diverse terpenoids.

BIOSYNTHESIS PATHWAYS OF DIVERSE TERPENOIDS

Albeit structurally diverse, skeletons of terpenoids are composed of C_5 isoprene units which are successively assembled by biogenic isoprene rule. Precursors of the C_5 isoprene units are isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are synthesized by either mevalonate (MVA) pathway in eukaryotes, archaea and a few bacteria or 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in prokaryotes and plant plastids (Liao et al., 2016; Frank and Groll, 2017). MEP pathway starts from condensation of two glycolytic intermediates, pyruvate and glyceraldehyde-3-phosphate, and MVA pathway from condensation of acetyl-CoAs (**Figure 1A**). Both pathways require energies (ATP) and reductive powers [NAD(P)H] to proceed multi-enzymatic reactions to produce IPP and DMAPP. Given no consideration of energy balance, MEP pathway consumes 1 molecule of glucose per IPP with a carbon molar yield of 0.83, while the MVA pathway consumes 1.5 molecules of glucose per IPP with the lower yield of 0.56 (Eqs. 1, 2) (Schempp et al., 2018). In this scenario, MEP pathway requires 2 ATP and 2 NAD(P)H, while MVA pathway accompanies generation of 3 NAD(P)H. Thus, microorganisms need engineering to use both pathways to attain the carbon and energy balances, which results in a synergy between both pathways for production of terpenoids (Yang et al., 2016).

$$\textbf{MEP}: 1\ \text{Glucose} + 2\text{ATP} + 2\ \text{NAD(P)H} \rightarrow 1\ \text{IPP} + \text{CO}_2$$

$$(Y_{\text{IPP/Glucose}} = 0.83\ \text{C-mol/C-mol}) \quad (1)$$

$$\textbf{MVA}: 1.5\ \text{Glucose} \rightarrow 1\ \text{IPP} + 4\ \text{CO}_2 + 3\ \text{NAD(P)H}$$

$$(Y_{\text{IPP/Glucose}} = 0.56\ \text{C-mol/C-mol}) \quad (2)$$

Next, prenyltransferases catalyze chain elongation of isoprenyl diphosphates including geranyl diphosphate (GPP, C_{10}), farnesyl diphosphate (FPP, C_{15}), geranylgeranyl diphosphate (GGPP, C_{20}), and even longer chain isoprenyl diphosphates (LoPP, $>C_{45}$), which determine the primary diversity of terpenoids in the chain lengths. Thus, terpenoids are classified to hemiterpenoids (C_5), monoterpenoids (C_{10}), sesquiterpenoids (C_{15}), diterpenoids (C_{20}), triterpenoids (C_{30}), tetraterpenoids (C_{40}), and long chain isoprenyl products (**Figure 1A**).

Terpene synthases convert isoprenyl diphosphates to numerous terpenes via carbocationic reaction cascades and/or hybridization of carbon atoms, which generate a myriad of carbon skeletons containing several stereocenters and present their diversity in the structure (Christianson, 2017). The skeletons are further regio- and stereo-selectively decorated by many tailoring enzymes such as cytochrome P450s (P450s), acyltransferase, and glycosyltransferase, which may act in a combinatory manner and finally render various functions to isoprenoids (e.g., artemisinin, taxol and ginkgolide) (King et al., 2016). Therefore, the microbes, for which a good genetic engineering toolbox is available, can be engineered to synthesize valuable terpenoids by assembling of the precursor synthesis, isoprenyl chain elongation, structural rearrangement and tailoring reactions.

MICROBIAL HOSTS FOR TERPENOID PRODUCTION

As most terpenoids have been discovered in plants, an extraction from plants is a major source of commercialized terpenoids. However, the plant extraction is compromised by its low productivity due to a slow growth of plants, a low product content in plants, and a limitation in cultivating some species. Even though there is a biotechnological progress in transgenic plants, it still remains difficult to engineer plants for improved isoprenoids contents. Microbes grow fast and do not heavily rely on land/water resources. Thus, they possess a great potential for sustainable mass production of terpenoids over plants. Microbial hosts in industry are required to meet the criteria: (i) a large metabolic potential supporting efficient synthesis of products of interest with robust and fast cell growth, (ii) a well-understood metabolism and well-developed genetic tools (e.g., expression vectors), and (iii) a great capacity to grow on cheap carbon sources. Terpenoids synthesis relies on MEP or MVA pathway for the generation of building units, IPP and DMAPP. Therefore, two representative microbial hosts *E. coli* and *S. cerevisiae*, which are based on MEP and MVA pathways, respectively, have been used for production of diverse terpenoids.

The terpenoids natively produced in *E. coli* are limited to small amounts (e.g., quinones). The native MEP pathway could be less efficient in IPP and DMAPP supply for terpenoid production. *E. coli* has been engineered to improve IPP and DMAPP synthesis by augmenting bottleneck enzymes of MEP pathway or introducing heterologous MVA pathway. *S. cerevisiae* accumulates a large amount of ergosterol, which presents a potential of its MVA pathway for terpenoid production. *S. cerevisiae* has redox systems that allow cytochrome P450 to modify terpenoids skeleton, whereas *E. coli* has none. *S. cerevisiae* is superior to *E. coli* in synthesis of value-added terpenoids with complicated structures. *Yarrowia lipolytica* is another good yeast for production of terpenoids based on MVA pathway because of its abundant acetyl-CoA, the initial substrate of MVA pathway (Zhu and Jackson, 2015). Besides, carotenogenic *Rhodosporidium toruloides* is developed to produce terpenoids from lignocellulosic hydrolysates in that it exerts ability to utilize multiple carbons and tolerates high osmotic stress (Yaegashi et al., 2017; Sundstrom et al., 2018). As many terpenoids have been isolated from *Streptomyces* species (Koksal et al., 2012; Rinkel et al., 2017), the bacteria could also be a potential host for terpenoids production in the future (Phelan et al., 2015; Khalid et al., 2017).

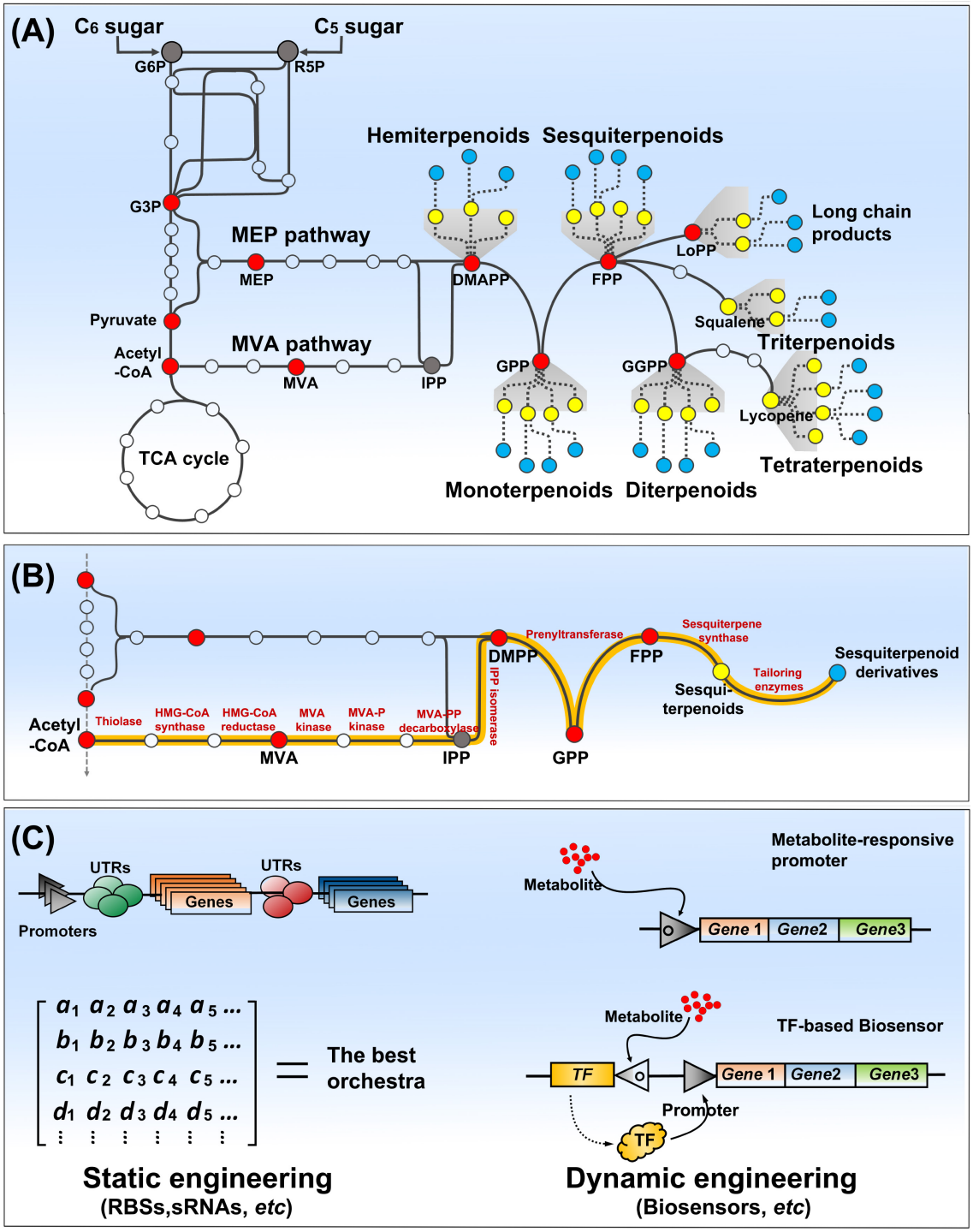

FIGURE 1 | Overview of terpenoids biosynthesis pathway **(A)**, and pathway engineering strategies **(B,C)**. Terpenoids biosynthesis is comprised of carbon assimilation, isoprene unit synthesis, terpenoids backbone synthesis, and terpenoids decoration. C_6 or C_5 sugar enters the central pathway of glycolysis. MEP and MVA pathways use central metabolites to initiate synthesis of IPP and DMAPP, the building blocks of terpenoids. Terpenoid synthesis pathways could be assembled and engineered in a tractable host (e.g., *E. coli* or yeasts) to create cell factories for their mass production. Two fashions of static and dynamic engineering have been used to optimize the synthesis pathways and coordinate them with host metabolic network. Static engineering approach generally constructs a matrix of genes, promoters, and regulatory elements (e.g., UTRs) to screen the best orchestra, while dynamic engineering approach relies on biosensors (metabolite response or transcription factor-based) to dynamically control the synthesis pathway. The red dots present the key intermediates of terpenoid biosynthesis, and the yellow and cyan dots present primary terpenenoids and decorated terpenoids, respectively **(A,B)**. TF presents transcription factor **(C)**.

METABOLIC ENGINEERING OF *E. coli* AND YEASTS FOR TERPENOID PRODUCTION

Microbial production of terpenoids can be addressed by introduction of relevant genes for their synthesis into host strains (**Figure 1B**). Owing to a versatility of *E. coli* and *S. cerevisiae*, many strategies of metabolic engineering have been developed and examined to increase terpenoid production in the both model hosts. The engineering strategies can be classified to "static" and "dynamic" according to a regulatory means of synthetic pathway. Both approaches have been applied to terpenoid synthetic pathways for an enhancement of IPP/DMAPP flux, a minimization of byproducts formation, a toxicity reduction of pathway intermediates, etc., Static engineering employs variation of vectors, promoters, ribosome binding sites (RBSs), and genes of interest, which are assembled in a plethora of biological systems, to optimize a synthesis of target products (Ren et al., 2017; **Figure 1C**). Brewer's yeast has been engineered by combinatory regulation of pathway genes to biosynthesize aromatic monoterpenes that impart hoppy flavor to beer (Denby et al., 2018). Farnesol is a desirable biofuel molecule derived from FPP by phosphatases. By variation of phosphatases, 526 mg/L of farnesol was produced in an engineered *E. coli* overexpressing a membrane integral phosphatase, PgpB (Wang C. et al., 2016). Further phosphatase mining retrieved a cytosolic phosphatase NudB, whose overexpression led to farnesol of 1.42 g/L along with hemiterpenoids isopentenol (Zada et al., 2018). Optimization of plasmids carrying synthesis pathway of terpenoids brought an enhanced production of monoterpenes, 653 mg/L of 1,8-cineole of and 505 mg/L of linalool, from *E. coli* (Mendez-Perez et al., 2017). Oleaginous *Y. lipolytica* is able to synthesize a massive amount of acetyl-CoA, and lipid droplet is supposed to store lipophilic terpenoids. Tuning of promoter strength by promoters shuffling resulted in β-carotene production of 111.8 mg/L in *Y. lipolytica* (Larroude et al., 2018).

However, the static engineering is too laborious to gain a desired performance from an engineered strain due to intermediates toxicity or metabolic burdens often occurring from a mass production of terpenoids, whereas the dynamic engineering can address such adverse circumstances. Thus, there is also a great interest to develop tunable or inducible promoters and small regulatory RNAs (Ghodasara and Voigt, 2017; Marschall et al., 2017; Portela et al., 2017; Trassaert et al., 2017), which could benefit to a dynamic control of synthetic pathways of terpenoids. Biosensors have been developed to sense small molecules (metabolites), which are applied to a transcription control of the committed pathway in response to metabolite abundance (Dekker and Polizzi, 2017). They are built to a simple metabolite responsive promoter or a transcription factor (TF)-based binary system (**Figure 1C**), which are incorporated as a metabolic controller for dynamic flux control (Mannan et al., 2017). For example, FPP repressive promoters were developed to down-regulate FPP biosynthesis and FPP activated promoters could be used to up-regulate expression of amorphadiene synthase converting FPP to amorphadiene. These two promoters were combined in *E. coli* to implement the negative and positive feedback loops of the synthesis and conversion of FPP, which is a critical metabolic node in amorphadiene synthesis. Amorphadiene production of 1.2 g/L was obtained from the engineered *E. coli*, which was a two-fold increase in the production through the dynamic control (Dahl et al., 2013). In *S. cerevisiae*, a large amount of FPP is mainly used for synthesis of ergosterol via squalene. Thus, squalene synthase (Erg9) condensing two FPPs to a squalene could be a critical regulation point to divert FPP flux to synthesis of terpenoids of interests. A dynamic control of Erg9 expression using ergosterol-responsive promoter could alter FPP flux to amorphadiene synthesis (Yuan and Ching, 2015). Biosensors have been developed to provide an additional exquisite regulatory means for tuning of synthetic pathway, and improved in both their dynamic responsive range and substrates spectrum (Rogers et al., 2016; Liu et al., 2017). Natural TFs (AraC and Gal4) were evolved to respond to IPP accumulation (Chou and Keasling, 2013). Their application in controlling zeaxanthin biosynthesis pathway resulted in a successful production of the carotenoid (C_{40}) at a titer of 722.5 mg/L (Shen et al., 2016). Synthetic biologists have also designed promoters responding to environmental signals such as pH and quorum sensing (QS) molecules (Tsao et al., 2010; Zargar et al., 2015; Rajkumar et al., 2016). It is capable control terpenoid biosynthesis with the environmental signals, not using expensive inducer molecules. A QS-based promoter system was successfully applied for an inducer free production of bisabolene from *E. coli*, where titer of bisabolene was increased to 1.1 g/L through optimization of LuxR and LuxI expressions (Kim et al., 2017).

GENOME-LEVEL ENGINEERING OF TERPENOIDS PRODUCTION

Rewiring of metabolic network of host strain is required for a full performance of an engineered pathway for product synthesis, when potential of the engineered production pathway is restricted due to a metabolic characteristics of the host. Many genetic tools are available for genome engineering in *E. coli* and yeasts. Homologous recombination is the most popular method for deletion and replacement of genetic parts. The glyceraldehyde 3-phosphate and pyruvate supply was rebalanced by tuning-down of glyceraldehyde 3-phosphate synthase, which led to a two-fold increase in lycopene production via the MEP pathway in *E. coli* (Jung et al., 2016). The central pathway was rewired in *S. cerevisiae* to synthesize acetyl-CoA with reduced losses of carbon and ATP. As acetyl-CoA was the substrate of the MVA pathway, rewiring of the central carbon metabolism in *S. cerevisiae* resulted in an increase in farnesene production by 25% along with a reduced requirement of oxygen by 75% (Meadows et al., 2016). Genome engineering tools have been developed to simultaneously target multiple genomic loci like as multiplex automated genome engineering (MAGE), and to precisely edit a specific genomic locus without a scar based on the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system (**Figure 2A**). MAGE was applied to

optimizing MEP pathway in *E. coli* for lycopene production (Wang et al., 2009). A modified method of MAGE was also successfully applied for β-carotene production in *S. cerevisiae* (Barbieri et al., 2017). As MAGE generates a vast of combinatorial variants in genome, it could be an effective genome engineering tool only when it is combined with a high-throughput screening (colorimetric or fluorescent) system enabling a selection of variants with desired traits. It is also difficult to incorporate large genetic parts (>1 kb) into genome by MAGE. CRISPR/Cas9 is currently powerful and popular genetic tool for precise genome editing (Jiang et al., 2013; Jessop-Fabre et al., 2016). The endonuclease Cas9 combined with guide RNA (gRNA) specifically recognizes a target site in genome, and facilitates site-specific nucleotide base-pair mutations, gene deletions, or large DNA fragment (around 8 kb at least) insertions (**Figure 2A**). β-Carotene synthesis pathway was integrated into *E. coli* genome by CRISPR/Cas9-based method. Both MEP pathway and central metabolic pathways were optimized in the engineered strain, and the final strain was cultured in fed-batch, yielding 2.0 g/L of β-carotene (Li et al., 2015). A set of genomic loci of *Y. lipolytica* have been identified for targeted marker-less integration using CRISPR/Cas9 (Schwartz et al., 2017). An automated platform for multiplex genome-scale engineering in *S. cerevisiae* has been also developed based on CRISPR/Cas9 (Si et al., 2017). Plasmid-based expression of production pathway has a problem of segregational or structural instability of plasmids, which becomes worse in case of inhibitory products to host cells. Moreover, expensive antibiotics is required to exert a selection pressure on host cells for prevention of plasmid loss, which increases a production cost. However, genome-based expression of production pathway could deliver stable and reproducible production with no use of antibiotics, which is important for mass scale production of industry. The top portion of MVA pathway was integrated into genomic loci of *adhE* and *ldhA* in *E. coli*, and *atpFH* genes were deleted to increase glycolytic rate. The genome-engineered strain exhibited both high productivity (∼1.01 g/liter/h) and yield (86.1% of theoretical yield) after 48 h of shaking flask culture (Wang J. et al., 2016). However, engineering of genome integrated production pathway is a cumbersome task compared to plasmid-based engineering. Plasmid is more easy and convenient to modulate genes dosage and to construct various genetic expression cassettes. The genome level engineering for optimization of production pathway was facilitated by CRISPR/Cas9-based convenient chromosomal promoters change, which is successfully applied for bisabolene synthesis in *E. coli* (Alonso-Gutierrez et al., 2018). A heterologous MEP pathway was integrated into genome of *S. cerevisiae* with endogenous MVA pathway. As the MEP pathway does not produce extra NADH which need oxidizing to maintain redox status, the engineered yeast enabled to rely solely on the MEP pathway instead of the MVA pathway for terpenoids biosynthesis under low aeration conditions (Kirby et al., 2016). MVA pathway genes attached with mitochondrial-targeting signal sequences were integrated into genome of *S. cerevisiae* to enable utilization of both cytosolic and mitochondrial acetyl-CoAs via the native cytosolic and the engineered exogenous mitochondrial MVA pathways. The engineered yeast produced 2.5 g/L of isoprene, an increase of 1.6-fold by the mitochondrial MVA pathway (Lv et al., 2016). A comprehensive understanding of whole metabolic networks is a prerequisite to rational editing of genomes or design of biosynthesis pathways (Campbell et al., 2017). Metabolic engineers paid efforts to interpret cellular phenomena at a systems level by measuring various cellular components including RNAs, proteins, and metabolites (**Figure 2B**). There are successful examples of terpenoid production driven by systems biology to debottleneck synthesis pathway (Alonso-Gutierrez et al., 2015; Li et al., 2017; Wada et al., 2017). An integrated approach of multi-level Omics data has been pursued to obtain a desirable phenotype of host strain for production, because a genetic manipulation may have positive impact at one metabolic layer but a negative or neutral impact at another (Goh and Wong, 2016; Lechner et al., 2016). IPP toxicity always challenges the engineered microbial systems for terpenoid production. By multi-level Omics-integrated analysis, formation of isoprenyl-ATP analog is recognized as a cause of the IPP toxicity, which suggests potential engineering strategies for terpenoids production from *E. coli* (George et al., 2018).

CONSORTIA PROCESS FOR PRODUCTION OF TERPENOIDS

Complicated structures of terpenoids are often a hurdle of the production using a single microbe. As *E. coli* is generally not a tractable host for P450 chemistry, it is difficult to produce terpenoids decorated by P450 from *E. coli*. Although an oxygenated taxanes is produced in *E. coli* with optimization of P450 expression and its reductase partner interaction (Biggs et al., 2016), it requires lots of elaborate endeavors and cannot be copied into an engineering of other P450s. It would be beneficial to engineer multiple organisms to carry out a complicated task together rather than an engineering of a single organism alone (Hays et al., 2015). With greater understanding of microbial traits, coordinated microbial consortia can be designed and built to address complex tasks. Expansion of metabolic capacities and improvement of production yields could be attained in the synthetic microbial communities along with simplification of engineering of a complicated long pathway to a few simple shorter pathways distributed in the community microbes. The synthetic microbial consortia can be classified an intraspecies homo-consortia and an interspecies hetero-consortia, of which each strain is assigned an individual role toward product synthesis (**Figure 2C**). A key issue of synthetic microbial consortia engineering is to balance each microbial population for a successful collaboration to attain the goal (Johns et al., 2016). In order to produce acetylated taxanes, the entire pathway was divided into taxadiene synthesis expressed in *E. coli* and P450 modification in *S. cerevisiae* (Zhou et al., 2015). *E. coli* was engineered to use xylose, but produce a wasteful acetate, while auxotrophic *S. cerevisiae* assimilated solely acetate for cell growth in presence of xylose as a sole carbon source. The mutualistic consortium could maintain a population balance of the co-cultured strains and doubled the

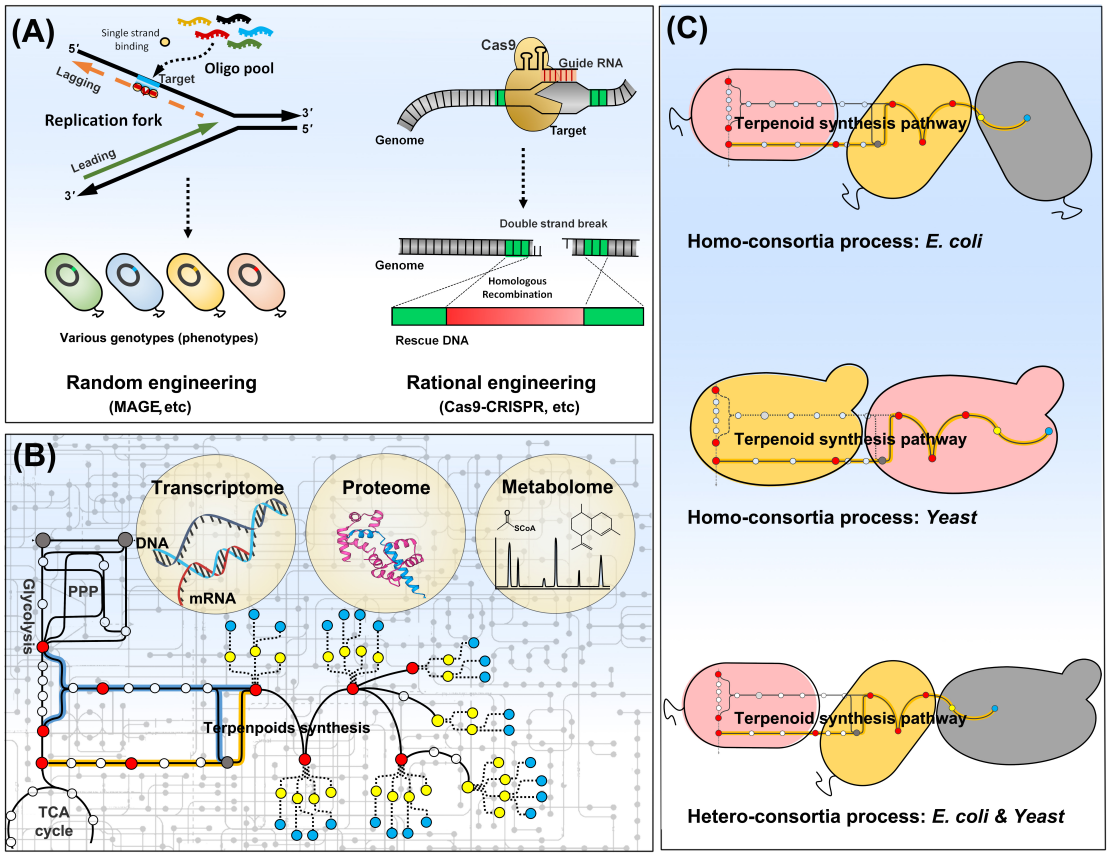

FIGURE 2 | Strain manipulation by genome editing **(A)**, integrated-omics **(B)**, and consortia process **(C)**. Host genome can be evolved by either random (e.g., MAGE) or rational engineering (e.g., Cas9-CRISPR). The integrated-omics approaches can comprehensively elucidate host metabolism, which benefit strain manipulation. The microbial consortia process divides a long complicated pathway into a few short simple pathways, dispersed among microbes in the consortia. It can be built in the same species (homo-consortia) or the different species (hetero-consortia).

production in comparison with a co-culture using glucose. Deeper understanding of autotrophy, interspecies cross-feed, and QS machinery would provide more exquisite approaches for production of complicated terpenoids using synthetic microbial consortia.

CONCLUSION

Currently, many enabling technologies of synthetic biology allow us to tailor microbes for terpenoids production. Among the microbial species, *E. coli*, and yeasts have proved as the most attractive microbial platform, which can be conveniently developed to generate either bulk or value-added terpenoids owing to versatile enabling technologies for the microbes. A great potential of *E. coli* and yeasts as platform strains has been demonstrated with many successes of synthetic pathway rewiring, genome editing, and microbial consortia building for improvement of production. With deeper understanding of their metabolism, more promising approaches are expected to boost production of terpenoids in *E. coli* and yeasts.

AUTHOR CONTRIBUTIONS

CW and S-WK developed the ideas and drafted the manuscript. ML, J-BP, and S-HJ collected the literatures and drew the figures. GW, YW, and S-WK professionally edited the manuscript.

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MINI REVIEW
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Recombinant Protein Expression System in *Corynebacterium glutamicum* and Its Application

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Corynebacterium glutamicum, a soil-derived gram-positive actinobacterium, has been widely used for the production of biochemical molecules such as amino acids (i.e., L-glutamate and L-lysine), nucleic acids, alcohols, and organic acids. The metabolism of the bacterium has been engineered to increase the production of the target biochemical molecule, which requires a cytosolic enzyme expression. As recent demand for new proteinaceous biologics (such as antibodies, growth factors, and hormones) increase, *C. glutamicum* is attracting industrial interest as a recombinant protein expression host for therapeutic protein production due to the advantages such as low protease activity without endotoxin activity. In this review, we have summarized the recent studies on the heterologous expression of the recombinant protein in *C. glutamicum* for metabolic engineering, expansion of substrate availability, and recombinant protein secretion. We have also outlined the advances in genetic components such as promoters, surface anchoring systems, and secretory signal sequences in *C. glutamicum* for effective recombinant protein expression.

Keywords: *Corynebacterium glutamicium*, expression host systems, cytosolic expression, secretory expression, recombinant protein, surface displayed expression

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INTRODUCTION

Recombinant proteins, including biologics and enzymes, are useful in the biopharmaceutical, food, and chemical industries (Butenas, 2013). To date, more than 400 recombinant biologics have been approved by the US Food and Drug Administration (FDA), and more recombinant biologics are in the clinical development stage (Sanchez-Garcia et al., 2016). The demand for new biologics (such as antibodies, growth factors, and hormones) for the treatment of severe chronic diseases (such as cancer, anemia, and multiple sclerosis) has increased, and the market for recombinant proteins is expected to grow over the next few decades (https://www.grandviewresearch.com/press-release/global-protein-expression-market). *Corynebacterium glutamicum*, which is a non-lethal and non-emulsifying gram-positive bacterium, exhibits a low protease activity in the culture supernatant and can secrete protease-sensitive proteins into the culture supernatant (Liu et al., 2013). Absence of lipopolysaccharide (endotoxin) in *C. glutamicum*, which is the gram-negative bacterial surface component that should be removed for the production of therapeutic proteins (Srivastava and Deb, 2005), may increase the heterologous protein yield by minimizing the purification steps. *C. glutamicum* has generally been used as a generally recognized as safe (GRAS) host for the industrial production of biochemicals including L-glutamate and L-lysine (Lee et al., 2016). As a result, *C. glutamicum* is favorable for producing high yields of proteins that are difficult to secrete in the host and the proteins that must remain active in a non-pathogenic environment.

The industrial production of biochemicals including nutraceuticals has been established using *C. glutamicum* as a host (Nakayama et al., 1961). Since *C. glutamicum* was first isolated as an L-glutamate producer by Kinoshita and Udaka in 1956 (Kyowa Hakko Bio Ltd. Co., Japan) (Kinoshita et al., 1957), many L-amino acids have been produced using this soil bacterium. In addition, many biochemicals (biopolymers, organic acids, rare sugars, etc.) have been commercially produced from metabolically engineered *C. glutamicum* strains. The metabolic processes of *C. glutamicum* may be rationally modified for the production of various biochemicals using three approaches: (1) amplification of biosynthetic pathway enzymes to increase target products, (2) reduction of by-product formation, and (3) introduction of important enzyme feedback controls to optimize target biomaterials. All these approaches involve the use of recombinant protein expression in the cytosol to produce beneficial biochemicals.

This review summarizes the recent studies on the heterologous expression of the recombinant protein in *C. glutamicum* for various applications including metabolic engineering, expansion of substrate availability, and recombinant protein secretion. It also lists the advancements of genetic components for effective recombinant protein expression.

CYTOSOLIC PROTEIN EXPRESSION IN *C. glutamicum* FOR METABOLIC ENGINEERING

A common method for producing biochemicals from *C. glutamicum* is the overexpression of enzymes involved in the biosynthetic pathway of the target product in cytosol (**Table 1**), which involves recombinant protein expression. Jensen and Wendisch overexpressed the ornithine cyclodeaminase (OCD) gene from *Pseudomonas putida* for the production of L-proline, which is a biochemical that is typically used as a commodity chemical or feed additive; this overexpression resulted in an increased product yield of 0.36 g proline/substrate (Jensen and Wendisch, 2013). Another foreign protein (D-lactate dehydrogenase) from *Lactobacillus delbrueckii* was expressed to address the limitations of using lactic acid bacteria, which require a relatively expensive complex medium for D-lactate production, and Okino et al. reported a high level of D-lactate production in *C. glutamicum* (Okino et al., 2008).

Jojima et al. designed protein expression systems as a way to reduce by-product formation in L-alanine production (Jojima et al., 2015). In a *C. glutamicum* strain, genes involved in the organic acid biosynthetic pathway (Δ*ldhA*: lactate dehydrogenase; Δ*ppc*: phosphoenolpyruvate carboxylase; Δ*alr*: alanine racemase) were inactivated; however, the *alaD* of *Lysinibacillus sphaericus* (encoding L-alanine dehydrogenase) along with the *gapA* of *L. sphaericus* (encoding glyceraldehyde 3-phosphate dehydrogenase promoting glucose consumption) were overexpressed, leading to a metabolic flux from organic acids to L-alanine. As a result, a high product (L-alanine) concentration (98 g/L_{medium}) was obtained.

As a large amount of oxygen and energy is required in the production of L-amino acids in *C. glutamicum* (Kwong and Rao, 1991), Liu et al. reported a novel approach for improving the intracellular oxygen supply by expressing hemoglobin (Liu et al., 2008). They modulated the metabolism to increase the productivity of L-glutamate by inducing metabolic flux into the tricarboxylic acid (TCA) cycle and additionally expressed the hemoglobin protein of *Vitreoscilla* sp. (VHb) in *C. glutamicum* to increase the oxygen and energy supply, resulting in the increased production of L-glutamine.

In addition, cytosolic protein expression in *C. glutamicum* has contributed to the production of biochemicals such as polyhydroxyalkanoate (PHA) (Matsumoto et al., 2011), ethanol (Jojima et al., 2015), and γ-aminobutyric acid (GABA) (Choi et al., 2015). The industrial techniques for the production of rare saccharides such as D-tagatose (Shin et al., 2016), D-sorbose and D-psicose (Yang et al., 2015), D-allose (https://patents.google.com/patent/WO2017111339A1/en), and GDP-L-fucose (Chin et al., 2013) are also the methods of nutraceutical production that involve cytosolic protein overexpression in *C. glutamicum*.

SURFACE-DISPLAYED ENZYME EXPRESSION IN *C. glutamicum*

Present Applications of Surface-Displayed Systems

The cell surface display systems can be used for a wide range of biotechnological and industrial applications: (1) a live vaccine that induces antigen-specific antibody responses by exposing heterologous epitopes to pathogenic bacterial cells (Lee et al., 2000); (2) screening of displayed peptides by sequential binding and elution (Boder and Wittrup, 1997); (3) expression of surface antigens to produce polyclonal antibodies in animals (Martineau et al., 1991); (4) using biological adsorbents for the removal of heavy metals (Bae et al., 2000); (5) using biological adsorbents for the removal of herbicides and environmental pollutants (Dhillon et al., 1999); (6) detecting single amino acid changes in target peptides after random mutagenesis; and (7) using biosensors with immobilized enzymes, receptors, or other signal-sensitive components (Aoki et al., 2002).

Purified enzymes have been used in many industrial bioconversion processes as immobilized enzyme catalysts. The immobilization of enzymes is time-consuming and costly because it involves several steps: growth of culture, disruption of cells, purification of enzymes, and immobilization of enzymes. When an enzyme is expressed on the cell surface of a microorganism as a whole-cell catalyst, additional purification steps are unnecessary and the whole-cell catalyst can be used repeatedly.

Development of Surface-Display Systems

The first surface expression system was developed in the mid-1980s to attach small peptides to proteins fused to bacteriophage surfaces (Smith, 1985). Thereafter, various phage display systems have been developed to express heterologous proteins on the surface of phages; however, the size of exogenous proteins that

TABLE 1 | Examples of cytosolic protein expressions in *Corynebacterium glutamicum* for productions of biochemicals.

Recombinant Protein	Product	Applications	Source	Producer	Titer (g/L_{medium})	Productivity (g/L/h)	Yield ($g_{product}/g_{substrate}$)	References
A. L-AMINO ACIDS AND RELATED BIOCHEMICALS								
Alanine dehydrogenase (AlaD)	L-Alanine	Supplement in animal feed	*Lysinibacillus sphaericus*	R Δ*ldhA* Δ*ppc* Δ*alr* + AlaD + GapA	98	3.1	0.83	Jojima et al., 2010
Glyceraldehyde 3-phosphate dehydrogenase (GapA)			*Corynebacterium glutamicum*					
Ornithine acetyltransferase (ArgJ)	L-Citrulline	Intermediate in the arginine biosynthesis, health, and nutrition applications	*Corynebacterium glutamicum*	ATCC 13032 Δ*argG* Δ*argR* + ArgJ	8.5	0.1	0.11	Zhang et al., 2018
Hemoglobin (Vgb)	L-Glutamine	Flavor enhancer	*Vitreoscilla*	ATCC14067 + GlnA (Y405F) + Vgb	17.3	0.36	0.08	Liu et al., 2008
3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DS), Chorismate mutase (CM), Prephenate dehydratase (PD)	L-Phenylalanine	Aromatic amino acids	*Corynebacterium glutamicum*	KY10865 + DS + CM + PD	28	0.35	0.47	Ikeda and Katsumata, 1992
Oornithine cyclodeaminase (ArgB)	L-Proline	Pharmaceutical and osmotic applications and feed additive	*Pseudomonas putida*	ATCC13032 Δ*argR* Δ*argF* + ArgB (A49V, M54V)	12.7	0.52	0.36	Jensen and Wendisch, 2013
Transketolase (TK)	L-Tryptophan	Supplement in animal feed	*Corynebacterium glutamicum*	KY9218 + DS + PGD + TK	58	0.73	0.25	Ikeda and Katsumata, 1999
3-eoxy-D-arabino-heptulosonate 7-phosphate synthase (DS), Chorismate mutase (CM)	L-Tyrosine	–	*Corynebacterium glutamicum*	KY10865 + DS + CM	26	0.32	0.43	Ikeda and Katsumata, 1992
B. ORGANIC ACIDS								
D-lactate dehydrogenase (D-LDH)	D-Lactate	Food packaging	*Lactobacillus delbrueckii*	R Δ*ldhA* + D-LDH	120	4	0.8	Okino et al., 2008
Glyoxylate reductase (YcdW)	Glycolate	Cosmetic industry to improve skin texture and to treat skin diseases	*Escherichia coli*	ATCC13032 Δ*aceB icd*GTG + YcdW	5.3	0.1	0.18	Zahoor et al., 2014
Cis-aconitate decarboxylase (CAD1)	Itaconic acid	Synthesis of resins, lattices, fibers, detergents, cleaners, and bioactive compounds	*Aspergillus terreus*	ATCC13032 *icd* A1G + MalE + CAD1 (optimized)	7.8	0.27	0.03	Otten et al., 2015
Acetohydroxy acid synthase (IlvBN), Acetohydroxy acid isomeroreductase (IlvC), Dihydroxy acid dehydratase (IlvD)	2-Ketoisovalerate	Precursor of L-valine, L-leucine, and pantothenate synthesis; substitute for L-valine or L-leucine in chronic kidney disease patients	*Corynebacterium glutamicum*	ATCC13032 Δ*ltbR* Δ*ilvE* Δ*prpC1* Δ*prpC2* + PgltA mut_L1 + IlvBN + IlvC + IlvD	35	0.79	0.15	Buchholz et al., 2013
Isopropylmalate synthase (leuA)	2-Ketoisocaprate	Therapeutic agent	*Corynebacterium glutamicum*	VB +IlvBN + IilvC + IlvD + leuA (G462D)	9.2	0.37	0.24	Bückle-Vallant et al., 2014
Alcohol dehydrogenase (ADH)	12-Ketooleic acid	Plasticizers, lubricants, detergents, cosmetics, and surfactants.	*Micrococcus luteus*	ATCC13032 + GFP + ADH	–	1.2	74%	Lee et al., 2015
C. POLYMERS								
Lysine decarboxylase (CadA)	Cadaverine	Replacement for the oil-derived hexamethylenediamine for polyamide 66 (nylon 66)	*Escherichia coli*	ATCC13032 Δ*hom* + AmyA + CadA	22.9 mM	–	–	Tateno et al., 2009

(Continued)

TABLE 1 | Continued

Recombinant Protein	Product	Applications	Source	Producer	Titer (g/L_{medium})	Productivity (g/L/h)	Yield ($g_{product}/g_{substrate}$)	References
Glutamate decarboxylase (GadB)	Gamma-aminobutyric acid (GABA)	Foods and pharmaceutical products	*Escherichia coli*	WJ008 + GadB mutant (Glu89Gln/Δ452-466 gene)	9.4	–	–	Choi et al., 2015
β-ketothiolase (PhaA), NADPH-dependent acetoacetyl-CoA reductase (PhaB), P(3HB) synthase (PhaC),	Poly-hydroxyalkanoate (PHA)	Alternative to plastics	*Ralstonia eutropha*	ATCC13869 + PhaA + PhaB +PhaC	6	–	–	Matsumoto et al., 2011
L-ornithine decarboxylase (SpeC)	1,4-Diaminobutane (putrescine)	Precursor of L-arginine and L-ornithine biosynthesis	*Escherichia coli*	ATCC13032 Δ*argR* Δ*argF* + SpeC + 5′21-ArgF (synthetic 5′-region)	19	0.55	0.16	Schneider et al., 2012
D. RARE SUGARS								
Rhamnulose-1-phosphate aldolase (RhaD)	D-Sorbose	Food additives, cancer cell suppressors, and building blocks for anticancer, and antiviral drug	*Escherichia coli*	SY6 + RhaD + YqaB (*lac* promoter)	19.5	–	–	Yang et al., 2015
Fructose-1-phosphatase (YqaB)	D-Psicose	Food additives, cancer cell suppressors, and building blocks for anticancer, and antiviral drug	*Escherichia coli*	SY6 + RhaD + YqaB (lac promoter)	13.4	–	–	Yang et al., 2015
D-galactose isomerase (D-Gal)	D-Tagatose	Functional sweetener	*Geobacillus thermodenitrificans*	PICG (Permeabilized and immobilized) + D-Gal	165	55	0.55	Shin et al., 2016
GDP-D-mannose-4,6-dehydratase (Gmd), GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase (ManB), Phosphomanno-mutase (WcaG), GTPmannose-1-phosphate guanylyl-transferase (ManC)	Guanosine 50-diphosphate (GDP)-L-fucose	Precursor of fucosyl-oligosaccharides	*Escherichia coli*	ATCC13032 Gmd + WcaG + ManB + ManC	0.086	0.001	–	Chin et al., 2013
E. ALCOHOL								
Pyruvate decarboxylase (Pdc), Alcohol dehydrogenase (AdhB)	Ethanol	Alternative transportation fuel	*Zymomonas mobilis*	R Δ*ldhA* Δ*ppc* + Pgi + PfkA + GapA + Pyk + Glk + Fba + Tpi + Pdc + AdhB	119	2.3	0.48	Jojima et al., 2015

can be expressed on the surface is limited (Li, 2000). For example, foreign proteins that can be expressed in M13 phages have a length of 6.6 nm (Lee et al., 2012), while most enzymes are more than 10 nm in diameter (http://book.bionumbers.org/how-big-is-the-average-protein).

To address this problem, microbial cell surface display systems have been developed (i.e., *C. glutamicum* is 2,000–6,000 nm in length and 500 nm in diameter). Microbial cell surface display is generally accomplished by expressing a passenger protein on the cell surface fused with preexisting microbial surface proteins or with anchoring motifs of the membrane protein (**Figure 1**). A C-terminal fusion, N-terminal fusion, or sandwich fusion strategy can be considered, depending on the characteristics of the fixed motif and the target protein. A good anchoring motif should meet the following requirements: (1) the premature fusion protein must have an efficient signal peptide or transport signal to pass through the inner membrane, (2) an anchoring motif must have a strong immobilization structure to retain the fusion protein on the cell surface, (3) an anchoring motif must be compatible with the inserted or fused foreign sequences (i.e., the anchoring motif should not become unstable following the insertion or fusion of heterologous sequences), and (4)

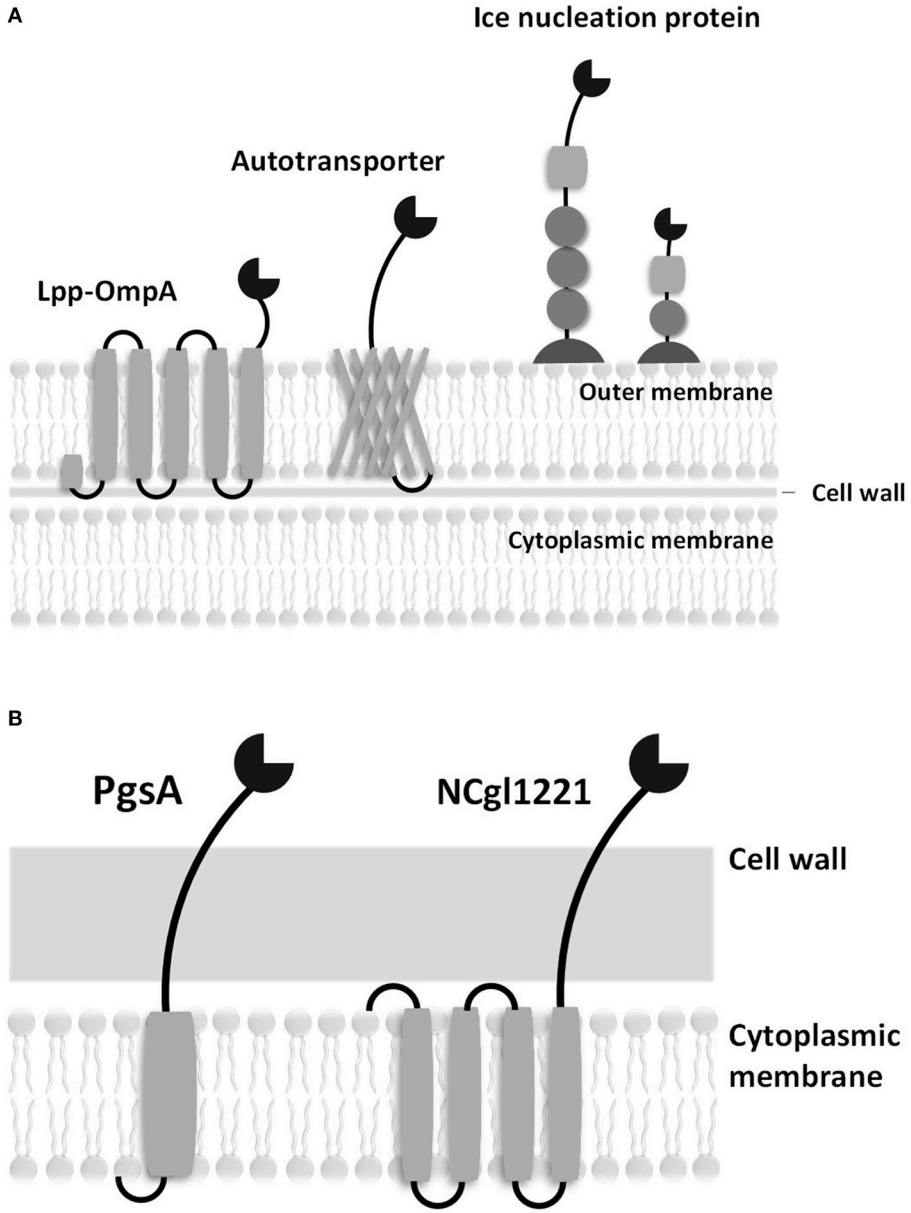

FIGURE 1 | Schematic diagrams of surface displayed enzymes in bacteria. **(A)** Surface display systems of gram-negative bacteria. **(B)** Surface display systems of gram-positive bacteria.

an anchoring motif must be resistant to attack by proteases present in the periplasmic space or media. In cell display systems, the properties of the target protein are known to significantly affect transport to the cell surface. In particular, the folded structure of the target protein (e.g., a disulfide bridge) in the outer membrane (the periplasmic side) can affect the movement of the target protein (Maurer et al., 1997). In addition, the insertion of amino acid sequences that contain multiple charged residues or hydrophobic residues within the target protein can result in ineffective sequence secretion in bacterial hosts.

Good hosts for surface display should be compatible with the expressed protein and should be easily culturable without cell lysis. In addition, the host cells must have a low extracellular protease activity. Gram-negative bacteria, including *Escherichia coli*, have a complex surface structure, which consists of the cytoplasmic membrane, periplasm, outer membrane, and many anchoring motifs that have been developed based on the outer membrane proteins. Therefore, the anchoring motif fused with the target protein in gram-negative bacterial hosts must be transferred to the outer membrane through the cytoplasmic membrane and periplasm. On the other hand,

gram-positive bacteria are considered to be more suitable for whole-cell catalysts and whole-cell adsorbents because of the robust structure of their cell walls. Many surface proteins can be covalently immobilized on the cell walls of *Bacillus* spp., *Staphylococcus* spp., and *C. glutamicum*. A eukaryotic GRAS host, *Saccharomyces cerevisiae*, has protein folding and secretion systems that are similar to those in mammalian cells; it has been reported that mammalian proteins could be linked to the cell wall via a glycosylphosphatidylinositol (GPI) anchor or disulfide bonds (Lee et al., 2003).

Examples of Surface-Displayed System in *C. glutamicum*

The use of *C. glutamicum*, an important industrial biochemical producer and as a Gram-positive bacterial host, is advantageous in a cell surface display system because of the presence of various enzymes on the surface of *C. glutamicum* cells that extend the range of carbon sources during the production of biochemicals (**Table 2**). Starch is used as an industrial carbon source for microorganisms; however, *C. glutamicum* cannot consume starch directly. Starch should be provided in a hydrolyzed form using α-amylase or glucoamylase. Tateno et al. have described the use of starch as a carbon source directly by a surface-displayed enzyme on *C. glutamicum* (Tateno et al., 2007). PgsA, a transmembrane protein derived from *Bacillus subtilis*, is a part of the poly-γ-glutamate synthetase complex. It was used to anchor the α-amylase from *Streptococcus bovis* 148 on the cell surface. The resulting display system was able to produce L-lysine (yield of 6.04 g/L_{medium}) from starch. In addition, a system displaying α-amylase fused with PgsA as an anchor produced 6.4% of poly-β-hydroxybutyrate (PHB) (Song et al., 2013) in a metabolically engineered *C. glutamicum*, using starch as raw material.

In addition to PgsA, porin has been used as an anchor protein in *C. glutamicum*. Porin is a cell wall-related protein of *C. glutamicum* that is present in the mycolic acid layer. Adachi et al. have produced 1.08 g/L_{medium} of L-lysine from a cellobiose carbon source using a system in which β-glucosidase was displayed using PorC as an anchor protein (Adachi et al., 2013). In addition, Imao et al. have reported the display of β-xylosidase on the cell surface using PorH as an anchor protein (Imao et al., 2017). In this expression system, xylooligosaccharides were used as a carbon source to produce 0.12 g/L_{medium} of 1,5-diaminopentane (cadaverine).

The use of other anchor proteins has also been reported by Yao et al. They displayed *S. bovis* 148 α-amylase on the cell surfaces using the C-terminally truncated NCgl1221 anchor protein. In this system, 19.3 g/L_{medium} of L-glutamate was produced from starch (Yao et al., 2009).

Recent reports of Choi et al. suggested that the proteins from 19 known mycolic acid layers in the extracellular membrane of *C. glutamicum* can be used as anchoring motifs in surface display systems (Choi et al., 2018). The α-amylase of *S. bovis* was screened using a portion of NCgl1337 as an anchoring motif; this portion has a signal peptide and a predicted O-mycoloylation site. As a result, 10.8 g/L_{medium} of L-lysine was obtained from starch; this result demonstrates the potential of whole-cell biotransformation using the cell membrane proteins of *C. glutamicum*.

SECRETIONS OF PROTEINS FROM *C. glutamicum*

Characteristics of Natural Secretion Systems in *C. glutamicum*

The direct secretion of proteins into the culture medium by *C. glutamicum* over protein expression in the cytosol has several advantages. First, it is easy to obtain the target protein by purification because it does not require cell disruption and there are fewer proteins in the culture medium than in the cytoplasm (Nguyen et al., 2007). In addition, the oxidative environment of the extracellular culture fluid is suitable for the formation of disulfide bonds, which leads to protein folding and the expression of the active protein form (Makrides, 1996). Furthermore, the low extracellular protease activity of *C. glutamicum* contributes to the stability of the target proteins (Suzuki et al., 2009).

Two major translocation pathways have been known identified in *C. glutamicum*: the secretory (Sec)-pathway and the twin-arginine translocation (Tat)-pathway. The Sec-pathway transports unfolded proteins, whereas the Tat-pathway transports folded proteins (Kudva et al., 2013). These two pathways have the signal peptides necessary for the protein to pass through the cell membrane (von Heijne, 1985). The difference between the signal peptides of the two pathways is that the N-region of the Tat-type signal peptide is longer than that of the Sec-type signal peptide because the Tat-type signal peptide contains a conserved twin-arginine residue (RR) at the end of the N-region (Berks et al., 2000; **Figure 2A**).

The Sec-pathway is a system that secretes proteins in an unfolded state (**Figure 2B**). Sec-dependent protein secretion systems have a co-translational targeting system and a post-translational targeting system (Fröderberg et al., 2004). In the co-translational targeting system, the signal recognition particle (SRP) binds to the nascent peptide and leads the complex (nascent peptide + ribosome) to a membrane protein FtsY along with ribosomes; then, this SPR subsequently leads the nascent peptide to the channel complex (SecYEG). In the post-translational targeting system, the translation-finished peptide binds to SecB and SecA to reach the SecYEG channel (Singh et al., 2014). Once the linear peptide passes through the SecYEG channel, the signal peptide is cleaved by Type I signal peptidase and the protein is released from the membrane (Schallenberger et al., 2012).

The Tat-pathway is a twin-arginine translocation pathway with a conserved twin-arginine motif (RR) in the signal peptide (**Figure 2C**). The basic structure of the Tat system is divided into two complexes: a docking complex and a pore complex. The docking complex (TatB and TatC) recognizes the RR motif of the Tat signal peptide in the folded protein. Then, the folded protein is translocated across the active pore complex (TatA), with a structural change of the docking complex (Goosens et al., 2015).

TABLE 2 | Examples of surface-displayed enzyme expressions in *Corynebacterium glutamicum* for expansion of substrate availability.

Passenger protein	Anchor protein	Substrate	Product	Resource	Producer	Titer (g/L_{medium})	Productivity (g/L/h)	Yield ($g_{product}/g_{substrate}$)	References
FOR AMINO ACIDS PRODUCTION									
α-amylase (AmyA)	PgsA	Starch	L-Lysine	AmyA: *Streptococcus bovis* 148 PgsA: *Bacillus subtilis*	ATCC13032 Δ*hom* + PgsA + AmyA	6.04	0.25	0.18	Tateno et al., 2007
α-amylase (AmyA)	NCgl1221	Starch	L-glutamate	AmyA: *Streptococcus bovis* 148 NCgl1221: *Corynebacterium glutamicum*	ATCC13869 + NCgl1221 + AmyA-FLAG	19.3	0.74	0.64	Yao et al., 2009
β-glucosidase (Sde1394)	Porin (*porC*)	Cellobiose	L-Lysine	β-glucosidase: *Saccharophagus degradans*	ATCC13032 Δ*hom* + PorC + Sde1394-FLAG	1.08	0.01	0.05	Adachi et al., 2013
β-glucosidase (Sde1394)	Porin (*porC*)	Cellobiose	L-Lysine	β-glucosidase: *Saccharophagus degradans*	DM 1729 + PorC + Sde1394	0.73	0.01	0.03	Anusree et al., 2016
α-amylase (AmyA)	Short-length (1–50) NCgl1337	Starch	L-Lysine	AmyA: *Streptococcus bovis* 148	ATCC13032 + NCgl1337 (Full length) + AmyA	10.8	0.6	0.29	Choi et al., 2018
FOR POLYMERS PRODUCTION									
α-amylase (AmyA)	PgsA	Starch	Polyhydroxybutyrate (PHB)	AmyA: *Streptococcus bovis* 148 PgsA: *Bacillus subtilis*	ATCC13032 Δ*hom* +PgsA + AmyA + PhaC + PhaA + PhaB	6.4wt%	0.88	1.6	Song et al., 2013
β-xylosidase (Xyl)	PorH	Xylooligosaccharides	1,5-diaminopentane (cadaverine)	*Bacillus subtilis*	PIS8 + PorH +Xyl + XylAB (*E. coli*) + ldcC	0.12	–	0.01	Imao et al., 2017
FOR ORGANIC ACIDS PRODUCTION									
α-amylase (AmyA)	PgsA	Starch	Lactate Succinate Acetate	AmyA: *Streptococcus bovis* 148 PgsA: *Bacillus subtilis*	ATCC13032 + PgsA + AmyA	6 1.5 0.7	0.6 0.15 0.07	0.65 0.16 0.07	Tsuge et al., 2013

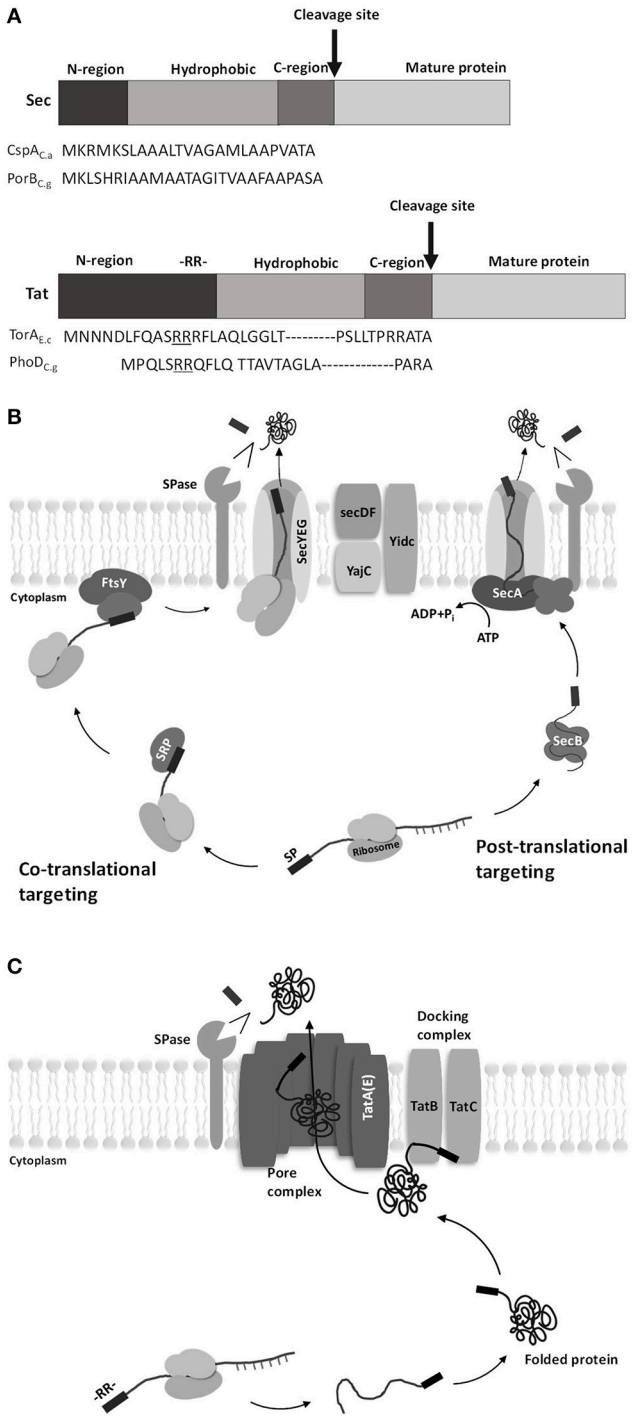

FIGURE 2 | Diagrams of signal peptides for Sec pathway and Tat pathway in *C. glutamicum*. **(A)** General structure and amino acid sequence of the Sec- and Tat-type signal peptides. The signal peptide consists of three regions: the amino-terminal region (N-region), the hydrophobic region, and the carboxy-terminal region (C-region). The difference between the two pathways is that the N-region of the twin-arginine translocation (Tat)-type signal peptide is longer than the secretory (Sec)-type signal peptide because the Tat-type signal peptide contains a conserved twin-arginine residue (RR) at the end of the N-region [CspA$_{C.a}$, surface (S)-layer protein from *Corynebacterium ammoniagenes*; PorB$_{C.g}$, porinB from *C. glutamicum*; TorA$_{E.c}$, TMAO reductase from *Escherichia coli*; PhoD$_{C.g}$, alkaline phosphatase from *C. glutamicum*] (Berks et al., 2000). **(B)** Protein translocation by the Sec pathway. Sec translocase consists of the following components: SecYEG, a core protein in Sec translocase that forms the transmembrane protein-conducting channel (PCC), and SecDF, interacts with YajC to improve protein transport efficiency driven by the proton motive force (Scotti et al., 1999). In the co-translational targeting Sec pathway, signal recognition particles (SRPs) bind to the signal peptide at the beginning of translation where proteins are still bound to ribosomes. Then, the SRPs and the initial ribosomal protein (nascent protein) migrate to the SRP receptor and membrane protein FtsY and subsequently come in contact with SecYEG. The nascent protein passes through SecYEG while the ribosome is attached. In the post-translational targeting Sec pathway, a translation-finished protein binds to SecB without ribosome and then migrates to SecA-SecYEG complex. The delivered protein then passes through SecYEG while SecA is attached. **(C)** Protein translocation by the Tat pathway. The Tat system consists of TatA-like proteins (TatA, TatB, and TatE) and TatC (TatE seems to have the same function as TatA, though the difference is not clear yet). Translocation begins when the folded cargo proteins interact with the docking complex. The twin-arginine (RR) motif of the Tat signal peptide attaches to the signal peptide-binding loop of TatBC. The docking complex recognizes the cargo protein and inserts it into the membrane. TatA receives the cargo protein from the docking complex, and the cargo protein is translocated across the active pore complex. The signal peptide is then cleaved by type I signal peptidase, and the mature protein is separated from the cell membrane (Tuteja, 2005).

Examples of Recombinant Protein Secretion in *C. glutamicum*

The production of a recombinant protein in *C. glutamicum* by a protein secretion system (the secretion of α-amylase from *Bacillus amyloliquefaciens* using the Sec system) was first reported by Smith et al. (Smith et al., 1986). Subsequently, protease, transglutaminase, green fluorescent protein (GFP), subtilisin, and endoglucanase have been produced in the *C. glutamicum* secretion system (**Table 3**).

Some studies have shown that eukaryotic proteins (such as human or camelid proteins) and microbial proteins could be successfully expressed in *C. glutamicum*. Yim et al. produced 68 mg/L$_{medium}$ of a single-chain variable fragment (scFv) with anthrax toxin as an antigen in a Sec system by codon optimization using a strong promoter (Yim et al., 2014). When the same M18 scFv was expressed in *E. coli*, a slightly higher level of the protein was obtained (89.8 mg/L$_{medium}$). Nevertheless, the use of *C. glutamicum* may be safer for drugs such as antibodies because endotoxins are not produced by a GRAS host, unlike the case of *E. coli*, and the secreted proteins are stable because there is no extracellular protease activity. Gram-positive bacteria such as *C. glutamicum* have no outer membrane; thus, target proteins need to pass through only one membrane to move out of the cell (van Wely et al., 2001). In addition, yeast cells that can glycosylate proteins when producing full-length antibodies are mainly used. However, in contrast to the glycosylation system of mammalian cells, yeast cells have a mannose-rich glycosylation system; thus, they are often not suitable for use in medicine. In particular, post-translational modifications such as glycosylation in *Pichia pastoris* often lead to unexpected protein structure and function (Dai et al.,

TABLE 3 | Examples of protein secretions in *Corynebacterium glutamicum*.

Proteins	Secretion system/resource*	Resource	Producer	Secreted protein titer (g/L_{medium})	References
SEC SYSTEMS					
Subtilisin (AprE)	Native	*Bacillus subtilis*	AS019 +*aprE*	0.0005	Billman-Jacobe et al., 1995
Protease (BprV)	AprE/B.s.	*Dichelobacter nodosus*	AS019 + BprV	0.0025	Billman-Jacobe et al., 1995
Protease (SAM-P45)	CspA/C.a	*Streptomyces albogriseolus*	ATCC13869 + SAM-P45	78 U/L	Kikuchi et al., 2003
Transglutaminase (MTG)	CspA/C.a	*Streptomyces mobaraense*	ATCC13869 + MTG	0.235	Kikuchi et al., 2003
Human epidermal growth factor (hEGF)	CspA/C.a	Human	YDK010 + hEGF	0.156	Date et al., 2006
Endoxylanase (XynA)	Porin B (PorB)/C.g	*Streptomyces coelicolor*	ATCC 13032 + XynA	0.615	An et al., 2013
Singlechain variable fragment (scFv)	Porin B (PorB)/C.g	*Escherichia coli*	ATCC 13032 + M18 scFv (codon-optimized)	0.068	Yim et al., 2014
Fab fragment of Human anti-HER2	CspA/C.a	Human	ATCC 13032 + Fab (H+L)	0.057	Matsuda et al., 2014
Endoxylanase (XynA)	Cg1514/C.g	*Streptomyces*	ATCC 13032 + XynA	1.07	Yim et al., 2016
α-amylase (AmyA)	Cg1514/C.g	*Streptococcus bovis*	ATCC 13032 + AmyA	0.78	Yim et al., 2016
Camelid antibody fragment (VHH)	Cg1514/C.g	Camelid	ATCC 13032 + CAb	1.58	Yim et al., 2016
α-amylase (AmyE)	CgR_2070/C.g	*Bacillus subtilis*	14067 + AmyE	103.24 U/mg	Jia et al., 2018
TAT SYSTEMS					
Endoglucanase (Clocel3242)	TorA/E.c	*Clostridium cellulovorans*	ATCC 13032 + Clocel3242	0.178	Tsuchidate et al., 2011
GFP	CgR0949/C.g	*Aequorea coerulescens*	R + AcGFP1	0.058	Teramoto et al., 2011
Sorbitol-xylitoloxidase (SoXy)	TorA/E.c	*Streptomyces coelicolor*	ATCC13032 + SoXy	–	Scheele et al., 2013
α-amylase	TorA/C.g	*Bacillus licheniformis*	BL-1 + pBlAmyS	0.49	Lee et al., 2014

B.s, Bacillus subtilis; C.a, Corynebacterium ammoniagenes; C.g, Corynebacterium glutamicum; E.c, Escherichia coli.

2015). Nevertheless, *C. glutamicum* may be advantageous as a host for the expression of antibody fragments such as the scFv and Fab (antigen-binding fragment), which do not require glycosylation (Yim et al., 2014). Matsuda et al. produced 57 mg/L_{medium} of an Fab fragment of anti-human epidermal growth factor receptor 2 (anti-HER2) using the Sec-secretion system with a cell wall protein-deficient *C. glutamicum* strain (Matsuda et al., 2014). This was based on the formation of an intermolecular disulfide bond when the heavy and light subunits of anti-HER2 Fab fragments were present at the same time. In another study, Date et al. reported the production of 156 mg/L_{medium} of an active human epidermal growth factor (hEGF) with six cysteine residues that form three disulfide bonds, using the Sec-secretion system in *C. glutamicum* (Date et al., 2006). Therefore, *C. glutamicum* is an attractive secretory expression host for the production of medicinal proteins containing disulfide bonds as well as heterologous enzymes.

Efforts have also been made to introduce new signal peptides in *C. glutamicum*. An analysis of the secretion of *C. glutamicum* at high cell densities showed that the most abundant protein (51% of extracellular proteins) in the culture supernatant was a hypothetical protein encoded by *cg1514*. Using the promoter and signal peptide of the Cg1514 protein, three target proteins [endo-colanicol A, 1.07 g/L_{medium}; *S. bovis* α-amylase, 0.78 g/L_{medium}; camelid antibody fragment (VHH) for human lysozyme, 1.58 g/L_{medium}] were produced (Yim et al., 2016). These results suggest that Cg1514-derived expression and secretion signals may be particularly effective in the production of secretory proteins from *C. glutamicum*.

Although not as common as the Sec system, there have been attempts to secrete proteins such as GFP or α-amylase using the Tat system in *C. glutamicum*. In particular, the Tat system is sometimes necessary because protein folding and the insertion of some cofactors into the proteins must occur in the cytoplasm. As the Tat system can transduce the substrates in a

TABLE 4 | Examples of inducible and constitutive promoters in *Corynebacterium glutamicum*.

Promoter	Description	References
INDUCIBLE PROMOTERS		
P_{lacUV5}	IPTG inducible promoter	Brabetz et al., 1991
P_{tac}	IPTG inducible promoter	Billman-Jacobe et al., 1994
P_{trc}	IPTG inducible promoter	Kirchner and Tauch, 2003
P_{prpB}	Propionate inducible promoter	Lee and Keasling, 2006
$P_{aceA/aceB}$	Acetate-inducible promoter	Cramer et al., 2006
$P_{gntP/gntK}$	Gluconate inducible promoter	Letek et al., 2006
P_{CJ1OX2}	42°C inducible promoter	Park et al., 2008
P_{tac-M}	Derived from the tac promoter, IPTG inducible promoter	Xu et al., 2010
P_{malE1}, P_{git1}	Maltose, Gluconate inducible promoter	Okibe et al., 2010
P_{BAD}	Arabinose inducible promoter	Zhang et al., 2012
SPLs	Synthetic promoter libraries, IPTG-inducible	Rytter et al., 2014
P_{4-N14}	Engineering the endogenous SigB-dependent promoter toward enhanced activity, stationary-phase gene expression system	Kim et al., 2016
CONSTITUTIVE PROMOTERS		
P_{cspB}	Promoter of *cspB* gene, encoding glyceraldehyde-3-phosphate dehydrogenase	Peyret et al., 1993
P_{aprE}	Promoter of *Bacillus subtilis* subtilisin (*aprE*)	Billman-Jacobe et al., 1995
P_{180}	Isolated promoter from *Corynebacterium glutamicum* genome library	Park et al., 2004
P_{sod}	Promoter of *sod* gene, encoding superoxide dismutase	Becker et al., 2005
P_{dapA}	Promoter of *dapA* gene, not known to be prone to transcriptional control	van Ooyen et al., 2012
P_{porB}	Promoter of *porB* gene, encoding porin B in *Corynebacterium glutamicum*	An et al., 2013
P_{ilvC}	Promoter of *ilvC* gene, encoding ketol-acid reductoisomerase	Kang et al., 2014
P_{L10}, P_{L26}, P_{I16}, P_{I51}, P_{H30}, P_{H36}	Fully synthetic promoter library consisting of 70-bp random sequences in *Corynebacterium glutamicum*	Yim et al., 2013; Oh et al., 2015

TABLE 5 | Examples of expression vectors in *C. glutamicum*.

Vector	Size (kb)	Replicon	Copy number per cell	Selection marker	Promoter, regulatory gene	Induction conditions (Conc.)	References
pEKEx1	8.2	pBL1	10–30	Km^r	P_{tac}, $lacI^q$	IPTG (0.2 mM)	Eikmanns et al., 1991
pXMJ19	6.6	pBL1	10–30	Cm^r	P_{tac}, $lacI^q$	IPTG (1 mM)	Anglana and Bacchetti, 1999
pBKGEXm2	7.3	pBL1	10–30	Km^r	P_{tac}, $lacI^q$	IPTG (1 mM)	Srivastava and Deb, 2002
pCRA1	5.3	pBL1	10–30	Cm^r	P_{lac}	Constitutive	Nakata et al., 2003
pCRA429	4.3	pBL1	10–30	Cm^r	P_{tac}	Constitutive	Suzuki et al., 2009
pDXW-8	9.6	pBL1	10–30	Km^r	P_{tac}, $lacI^{PF104}$	IPTG (1 mM)	Xu et al., 2010
pEC901	8.5	pCG1	30	Km^r	P_L/P_R (λ), cI857	40°C	Makoto Tsuchiya, 1988
pZ8-1	7.0	pCG1	30	Km^r	P_{tac}	Constitutive	Dusch et al., 1999
pVWEx1	8.5	pCG1	30	Km^r	P_{tac}, $lacI^q$	IPTG (1 mM)	Peters-Wendisch et al., 2001
pSL360	6.5	pCG1	30	Km^r	P_{180}	Constitutive	Park et al., 2004
pECXK99E	7.0	pGA1	30	Km^r	P_{tac}, $lacI^q$	IPTG (0.5 mM)	Kirchner and Tauch, 2003
pTRCmob	6.4	pGA1	30	Km^r	P_{trc}	IPTG (0.2 g/L_{medium})	Liu et al., 2007
pAPE12	4.6	pNG2	<10	Km^r	P_{tac}, $lacI^q$	IPTG (0.15 g/L_{medium})	Guillouet et al., 1999

fully collapsed state through the cytoplasmic membrane, the use of the Tat-pathway for enzyme secretion has been investigated. To this end, FAD cofactor-containing sorbitol-xylitol oxidase (SoXy), which is a cytosolic enzyme of *Streptomyces coelicolor*, was expressed in *C. glutamicum* using the Tat secretion system (Scheele et al., 2013). This study demonstrated that heterologous proteins containing cofactors can also be produced using the *C. glutamicum* secretion system.

GENETIC TOOLS FOR PROTEIN EXPRESSION IN *C. glutamicum*

To express recombinant proteins efficiently for the amino acid, food, and pharmaceutical industries, it is necessary to precisely control the expression of genes and to optimally control the metabolic flow toward the target protein or amino acid. Therefore, to produce the target protein efficiently, it is important to do the following: (1) optimize the promoter to increase expression efficiency, (2) construct a plasmid vector for various kinds of proteins, (3) construct an efficient protein-secretion pathway, and (4) design a *C. glutamicum* bioreactor culture system for high-yield production.

There have been attempts to increase the yield of expression systems, by using promoters (**Table 4**), which are mainly used for the production of amino acids and industrial enzymes with *C. glutamicum* as a host. The selection of optimal promoter and regulatory sequences is essential for producing useful products in living organisms. Promoters that are mainly used in *C. glutamicum* include several inducible promoters such as P_{lacUV5}, P_{tac}, P_{trp}, P_{araBAD}, $P_{trc,}$ and the phage P_R/P_L promoter from *E. coli* (Rytter et al., 2014). However, due to the low isopropyl-β-D-thiogalactopyranoside (IPTG) permeability of *C. glutamicum*, an IPTG-inducible expression system would have a lower expression level in *C. glutamicum* than in *E. coli*. Therefore, studies have been carried out to improve the promoter core sequence and membrane permeability of *C. glutamicum* and to increase gene expression by the site-directed mutagenesis. For example, a single-site mutation of the wild-type lac promoter has been used to enhance its protein expression level (Brabetz et al., 1991). In addition, the tac-M primer for constructing the tac promoter was found to increase the promoter activity following a mutation at the −10 region (Xu et al., 2010).

An auto-inducible promoter is a promoter that expresses proteins according to variables such as the nutrient type/concentration, oxygen level, pH level, and cell growth stage (Chou et al., 1995); this is advantageous for producing recombinant proteins on an industrial scale. Kim et al. (2016) engineered the SigB-dependent cg3141 promoter in *C. glutamicum* to develop an auto-inducible promoter system that is capable of expressing recombinant proteins during the transition phase between the log phase and the stationary phase of the cells. As a result, the model protein, glutathione S-transferase, was successfully produced on a lab-scale bioreactor (5 L) by introducing the P_{4-N14} promoter (Kim et al., 2016).

The use of constitutive promoters is advantageous because they do not require expensive reagents for induction or optimized induction conditions (Yim et al., 2013). Constitutive promoters derived from the genome of *C. glutamicum*, such as P_{sodA}, P_{gapA}, P_{eftu}, and P_{cspB}, are known to have high expression levels. However, the strength of the promoters cannot be directly compared and the use of strong promoters can also be affected by other genetic elements such as the 5′-untranslated region (5′-UTR) (Teramoto et al., 2011) and transcription initiation region (TIR) (Yim et al., 2013). Therefore, the selection of an optimal promoter is required because a strong promoter alone does not guarantee high protein expression. Yim et al. have developed the first synthetic promoter in *C. glutamicum* (Yim et al., 2013). Sequences including P_{L10}, P_{L26}, P_{I16}, P_{I51}, P_{H30}, and P_{H36} were selected from the promoter library, which consisted of 70 randomly chosen nucleotide sequences. Among them, P_{H36} was the strongest promoter, and it successfully induced the expression of antibody fragments and endoxylanase (746 mg/L_{medium}), as model proteins. Appropriate expression vectors and promoters are also important for increasing the yield of recombinant proteins. Currently, several *C. glutamicum*–*E. coli* shuttle expression vectors are being widely used (**Table 5**).

CONCLUSION

C. glutamicum can be used as an industrial L-glutamate and L-lysine producer. In addition, various types of recombinant proteins can be expressed in *C. glutamicum*, which has been used for several decades for the production of microbial enzyme. Furthermore, *C. glutamicum* has been used to increase yields, develop new anchoring systems, and signal peptides (for the efficient production of biochemicals and nutraceuticals, enzymes, medicinal proteins, and biopolymers), and screen synthetic promoters of various strengths. However, using *C. glutamicum* as an expression host has several disadvantages when compared with using *E. coli* as an expression host: (1) a much lower transformation efficiency, (2) fewer available expression systems, and (3) lower yields for some proteins, especially antibodies. Therefore, further studies are necessary to develop various tools to enhance protein yields and reduce manufacturing costs. Recent advances in bioinformatics, such as next-generation sequencing (NGS), RNA-seq, and proteomics, would provide more information on the protein production pathways in *C. glutamicum*.

AUTHOR CONTRIBUTIONS

ML wrote the manuscript and PK supervised. All authors have made intellectual contributions to the work, and approved it for publication.

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Engineering of the Filamentous Fungus *Penicillium chrysogenum* as Cell Factory for Natural Products

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Penicillium chrysogenum (renamed *P. rubens*) is the most studied member of a family of more than 350 *Penicillium* species that constitute the genus. Since the discovery of penicillin by Alexander Fleming, this filamentous fungus is used as a commercial β-lactam antibiotic producer. For several decades, *P. chrysogenum* was subjected to a classical strain improvement (CSI) program to increase penicillin titers. This resulted in a massive increase in the penicillin production capacity, paralleled by the silencing of several other biosynthetic gene clusters (BGCs), causing a reduction in the production of a broad range of BGC encoded natural products (NPs). Several approaches have been used to restore the ability of the penicillin production strains to synthetize the NPs lost during the CSI. Here, we summarize various re-activation mechanisms of BGCs, and how interference with regulation can be used as a strategy to activate or silence BGCs in filamentous fungi. To further emphasize the versatility of *P. chrysogenum* as a fungal production platform for NPs with potential commercial value, protein engineering of biosynthetic enzymes is discussed as a tool to develop *de novo* BGC pathways for new NPs.

Keywords: *Penicillium chrysogenum*, natural products, nonribosomal peptides, polyketides, gene activation, biosynthetic gene clusters, cell factory

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INTRODUCTION

Since the discovery of penicillin by Alexander Fleming produced by the filamentous fungus *Penicillium notatum*, the genus *Penicillium* has been deeply studied for its capacity to produce a wide range of natural products (NPs) (secondary metabolites), many of them with biotechnological and pharmaceutical applications. *P. chrysogenum* (recently renamed as *P. rubens*) is the most relevant member of more than 354 *Penicillium* species that constitute the genus (Nielsen et al., 2017). *Penicillium* is usually found in indoor environments and associated with food spoilage. It is known as an industrial producer of β-lactam antibiotic in particularly penicillin, and current production strains result from several decades of classical strain improvement (CSI) (Gombert et al., 2011; Houbraken et al., 2011). The CSI program began in 1943 with the isolation of *P. chrysogenum* NRRL 1951 capable of growing in submerged cultures. This strain was subjected to a long serial process of mutations induced by 275 nm ultraviolet and X-ray irradiation, nitrogen mustard gas and nitroso-methyl guanidine exposure, single spore selection and selection for loss

of pigments, improved growth in large scale industrial fermenters and enhanced levels of penicillin production. CSI programs were developed in several companies (Barreiro et al., 2012), and this has resulted in an increase of penicillin titers by at least three orders of magnitude (van den Berg, 2010). As consequence, numerous genetic modifications were introduced in *P. chrysogenum*. Some have been studied in detail, most notably the amplification of the penicillin biosynthetic clusters and DNA inversions in this region (Fierro et al., 1995, 2006; Barreiro et al., 2012). Although the CSI had a major impact on the production of β-lactams by *P. chrysogenum,* it also affected secondary metabolism in general. Indeed, a proteome analysis performed between *P. chrysogenum* NRRL 1951 and two derived strains (Wisconsin 54-1255 and AS-P-78) showed reduced levels of proteins related to secondary metabolism in the higher penicillin producer strains (Jami et al., 2010). Genome sequencing of *P. chrysogenum* Wisconsin 54-1255 revealed the presence of several secondary metabolite encoding biosynthetic gene clusters (BGCs) in addition to the penicillin cluster, most of which have only be poorly studied and remain to be characterized (**Figure 1**). The products of the BGCs are either nonribosomal peptides (NRPs), polyketides (PKs) or hybrid molecules.

Penicillium chrysogenum produces a broad range of secondary metabolites such as roquefortines, fungisporin (a cyclic hydrophobic tetrapeptide), siderophores, penitric acid, ω-hydroxyemodin, chrysogenin, chrysogine, sesquiterpene PR-toxin and sorbicillinoids, but likely also possesses the ability to produce other compounds not detected before. For most of the identified compounds, the responsible BGCs are unknown. The development of new bioinformatics tools (SMURF, AntiSMASH) (Khaldi et al., 2010; Weber et al., 2015; Blin et al., 2017) and the increase in the number of fungal genomes sequenced to date has opened the possibility to discover new NPs with novel properties (genome mining). The genes involved in the biosynthesis, regulation and transport of secondary metabolites tend to be arranged in the genome in clusters. Importantly, these gene clusters include the core biosynthetic genes which either encode polyketide synthases (PKSs), nonribosomal peptide synthetases (NRPSs) or terpene synthases genes (Smanski et al., 2016). Recently, a global analysis was performed on 24 genomes of *Penicillium* species and this identified 1,317 putative BGCs predominated by two classes based on PKS (467) and NRPS (260) (Nielsen et al., 2017). In *P. chrysogenum* there are 33 core genes in the secondary metabolism that encode 10 NRPS, 20 PKS, 2 hybrid NRPS–PKS, and 1 dimethyl-allyl-tryptophan synthase (van den Berg et al., 2008; Khaldi et al., 2010; Medema et al., 2011; Samol et al., 2016) (**Figure 1**). A large number of PKS and NRPS enzymes are found also in other *Penicillium* species but only part of these gene clusters are shared, which suggests an unexplored potential of the secondary metabolome even in a single genus.

Here, we summarize the most recent strategies for engineering filamentous fungi with particular attention to *P. chrysogenum*, a promising cell factory of novel products with new application spectra. A brief description of the key biosynthetic enzymes involved in biosynthesis of secondary metabolites in fungi is provided.

THE BUILDING ENZYMES OF THE NATURAL PRODUCTS

Nonribosomal peptide synthetases are large, highly structured and complex enzymatic machineries, closely related to other modular enzymes such as PKSs, NRPS–PKS hybrid synthetases and fatty acid synthetases (FASs). They have certain distinct properties in common, the most striking one being their structural division in domains and modules, which is manifested in their shared evolutionary history (Smith and Sherman, 2008). Every enzyme minimally consists of one module, a functionally distinct unit, which allows for the recruitment and subsequent incorporation of a precursor into a growing product. Domains as well as modules are clearly defined and evolutionary exchangeable structures amongst multi-modular enzymes. In the case of PKS and NRPS, this led to the occurrence of a variety of NRPS–PKS hybrids (Du and Shen, 2001; Shen et al., 2005; Li et al., 2010; Nielsen et al., 2016).

Nonribosomal Peptides (NRPs) and Nonribosomal Peptide Synthetases (NRPSs)

In comparison to most ribosomally derived peptides, NRPs are low molecular weight products. The structural diversity of NRPs is tremendous, mostly due to their chemical complexity. Significantly contributing to this diversity is the fact that NRPS are not only reliant on proteinogenic amino acids, but up until now more than 500 substrates were identified, which serve as NRPS building blocks (Caboche et al., 2008). These molecules are predominantly amino acids, but not exclusively, since fatty acids, carboxylic acids and others substrates have been reported in NRPs (Marahiel et al., 1997). NRP thus represent a diverse group of natural compounds and occur as linear, branched, circular or macrocircular structures (Dang and Sussmuth, 2017; Sussmuth and Mainz, 2017). The natural functions of NRPs are as diverse as their structures. Signaling, communication, metal-ion chelation, host protection are important functions performed by NRPs, though many compounds are not yet fully characterized in this respect. Nevertheless, the characterization of NPs for applied purposes is well developed and led to a vast collection of ground-breaking pharmaceuticals, including antibiotics, anti-fungal agents, immunosuppressants as well as cytostatic drugs (Frisvad et al., 2004; Watanabe et al., 2009; Dang and Sussmuth, 2017).

Structurally, every NRPS module, initiation (1), elongation (n) or termination (1), requires a minimal set of domains (**Figure 2A**) (Stachelhaus and Marahiel, 1995). The two domains essential to every module are the adenylation domain (A) and the non-catalytic thiolation domain (T). This tandem di-domain enables the specific selection and activation of a given substrate. However, the T-domain must first go through 4′-phosphopantetheinyl transferase (PPTase) and coenzyme A (CoA) dependent activation after expression, by transferring the phosphopantetheine moiety of CoA onto a conserved serine residue, in order to enter the holo state. Also, adenylation domains (A) have accompanying factors, or proteins, called

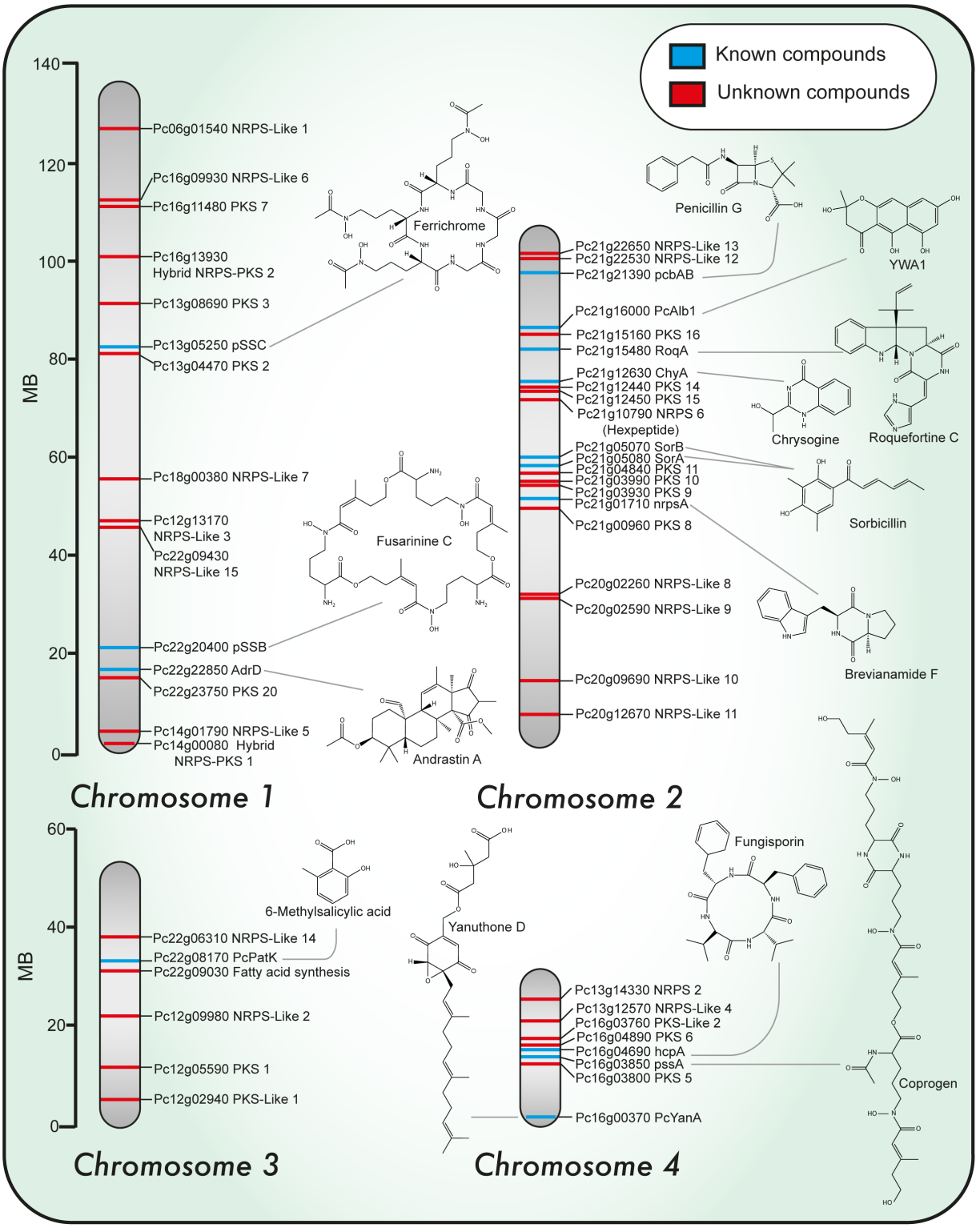

FIGURE 1 | Chromosomal localization of known and predicted PKS and NRPS genes and representative structures of associated secondary metabolites identified in *Penicillium chrysogenum*. Chromosomal localization of PKS and NRPS genes. Blue and red lines indicate known and unknown associated products so far, respectively.

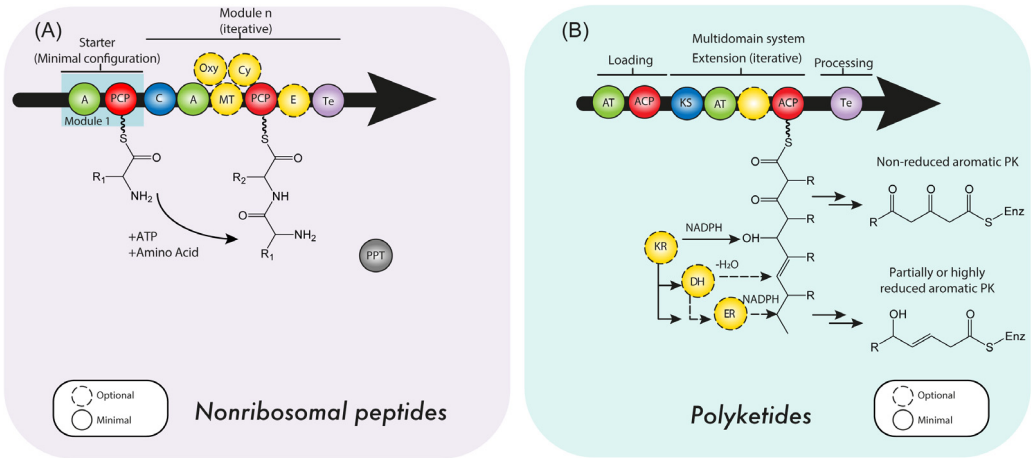

FIGURE 2 | (A) Nonribosomal peptide synthetases (NRPSs) and **(B)** polyketide synthase (PKS) minimal domain structure. For details see the text. C, condensation domain; PCP, peptidyl carrier protein; A, adenylation domain; E, epimerase; MT, methyltransferase; PPT, 4'-phosphopantetheine transferase domain; Oxy, oxygenation domain; Cy, cyclization domain; ACP, acyl carrier protein; AT, acyltransferase domain; KS, ketosynthase domain; KR, ketoreductase domain; DH, dehydratase domain; ER, enoyl reductase domain; Te, thioesterase domain.

MbtH-like proteins (MLPs) (Quadri et al., 1998; Baltz, 2011). In contrast to PPTases, MLPs are merely interacting with the A-domain, however, they do not have an intrinsic enzymatic activity, but rather a chaperoning function upon binding a distinct part of the A domain (Felnagle et al., 2010; Miller et al., 2016; Schomer and Thomas, 2017). In addition to these domains, any elongation module will require a condensation domain (C), which connects two modules and links up- and downstream activated substrates via a peptide bond. C-domains are stereospecific for both, up- and downstream activated substrates and render the resulting intermediate compound attached to the downstream T-domain. Lastly, the C-terminal termination module essentially requires a thioesterase domain (Te), to catalytically release the covalently bound compound of the NRPS, returning the NRPS complex to the ground state for another reaction cycle. In addition to these essential domains, we can distinguish a series of additional domains, performing epimerization, halogenation, cyclization, macrocyclization, multimerization or methylation (Ansari et al., 2008; Horsman et al., 2016; Bloudoff and Schmeing, 2017).

Theses enzymatic machineries can be classified as *type I NRPS* when the modules are arranged on a single protein, while the *type II NRPS* are independent proteins in an transient manner during the NRP synthesis (Sattely et al., 2008; Hur et al., 2012). A NRPS can be as simple as a single modular unit containing three domains, although the most complex and largest structure known contains 15 modules with 46 domains (Wang H. et al., 2014; Bode et al., 2015) yielding a 1.8 MDa protein complex (type I NRPS). Although the size of a NRPS, as well as the modular sequence, limits the setup of the resulting NRP, it is common that NRPS cluster and interact with tailoring enzymes in order to produce products of a higher complexity (Yin and Zabriskie, 2006). To enable such specific interactions, NRPS can contain small stretches of up to 30 amino acids at the C- or N-terminus, which form a rather specific recognition point, thus enabling communication (COM-domain) between multiple NRPS of one cluster (type II NRPS) (Hahn and Stachelhaus, 2006; Dowling et al., 2016). To date three types of NRPS system have been described according to their synthesis mode (or strategy of biosynthesis): Type A (linear), type B (iterative) and type C (non-linear) (**Figure 3**). The ***type A system*** harbors the typical domain organization A-T(C-A-T)$_{n-1}$-Te/C, where n represents the number of amino acids in the peptide. In this linear NRPS, the order and number of modules correlates with the amino acid sequence in the NRP and thus it is possible to predict the product that will be formed. Usually, in fungal NRPSs the cyclisation reaction is performed by a specialized C domain instead of Te domain. Since each module catalyzes one cycle during the chain elongation of the nascent NRP due to its specific activity, this system is considered analogous to type I PKS. In fungi, the most prominent examples of this type of NRPS are ACV synthetases (β-lactams), cyclosporin synthetases (Cyclosporin A) and peptaibol synthetases (peptaboils, a class of antibiotics with a high content of α-aminoisobutyric acid) (Wiest et al., 2002; Tang et al., 2007; Felnagle et al., 2008; Eisfeld, 2009). The ***type B system*** is characterized to employ all their modules or domains more than once during the synthesis of a single NRP, which enables the assembling of peptide chains that contain repeated sequences along the structure (Mootz et al., 2002). An example of this mode of synthesis occurs in *Fusarium scirpi* during the biosynthesis of enniatin (antibiotic), which is achieved through the repeated use of two modules. Other examples of type B NRPSs are the siderophore synthetases, which only contain three A domains that catalyze the biosynthesis of ferrichrome (Mootz et al., 2002; Eisfeld, 2009). In ***type C system***, the non-linear NRPSs have at least one domain conformation that deviate from (C-A-T)$_{n-1}$ organization contained in linear NRPSs. Likewise, in these synthetases the module arrangement does not correspond to the amino acid sequence in the NRP. Unlike type A NRPS, in type C NRPSs the non-linear peptide is

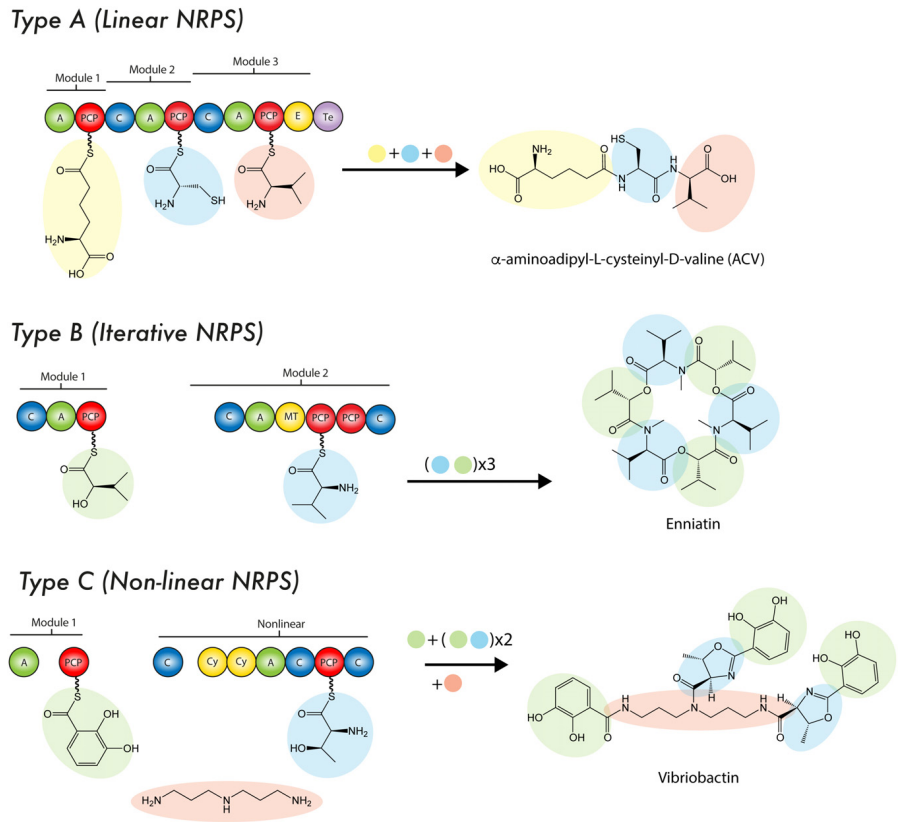

FIGURE 3 | Mode of biosynthesis of nonribosomal peptide synthetases. For details see the text. C, condensation domain; PCP, peptidyl carrier protein; A, adenylation domain; E, epimerase; MT, methyltransferase; Te, thioesterase domain; Cy, cyclization domain.

produced by a branch-point synthase and contains uncommon cyclization patterns. Another important difference is that non-linear NRPSs can incorporate small soluble structures, such as amines into the rising NRP through specialized C domains (Tang et al., 2007; Felnagle et al., 2008; Hur et al., 2012). Capreomycin, bleomycin and vibriobactin are examples of NRPs produced by this type of synthetases (Felnagle et al., 2008). In continuation, a brief description of the main NRPS domains features is provided.

Adenylation (A) and Thiolation (T) Domains

Any NRPS module minimally consists of an A- and T-domain (or peptidyl carrier protein, PCP), enabling single module functionality and multi-modular functionality upon addition of C domains (Linne and Marahiel, 2000; Bergendahl et al., 2002; Felnagle et al., 2008; Kittilä et al., 2016; Bloudoff and Schmeing, 2017). They are often referred to as "gatekeeper" domains, as there is no subsequent product formation without prior adenylation and thioesterification of a substrate (Sun et al., 2014). The two core functions of the A-domain are characterized first, through the hydrolysis of ATP or adenylation, allowing an AMP-substrate conjugate to be formed, which is subsequently transferred to the free thiol group of the 4′-phosphopantetheinyl-moiety (Ppant), which is anchored to a conserved serine residue in the downstream T-domain (Ku et al., 1997; Weber and Marahiel, 2001; Neville et al., 2005).

Condensation Domains (C)

C-domains are approximately 450 residue NRPS domains, representing a highly versatile class of NRPS domains. Any NRPS composed of more than one module must consequently contain at least one C-domain. However, also single modular NRPS may contain C-domains, especially if they cooperate with other NRPS. Essentially, the primary target of a C-domain is the condensation of the up- and downstream activated substrates through a nucleophilic attack, mainly leading to the formation of an n-peptide linked via a peptide bond. Nonetheless, several residues of the C-domain may have the intrinsic potential to fulfill multiple functions (Balibar et al., 2005; Teruya et al., 2012; Haslinger et al., 2015).

Epimerization Domains (E)

The E-domains are among the most abundant modification domains intrinsic to NRPS. In contrast to the structurally similar C domains they are responsible for the site specific epimerization of a substrate, predominantly performing this function after peptide bond formation has occurred (Bloudoff and Schmeing, 2017).

Thioesterase Domains (Te)

The thiotemplate based enzymatic systems rely on a catalytic activity in order to remove a product or product-scaffold of the primary enzyme. Therefore, most NRPS contain a domain on their C-terminus responsible for precisely this purpose, the thioesterase domain (Te). Te-domains are a common commodity in single and multi-modular NRPS, although, in multi-NRPS systems only the terminal NRPS contains this domain (Horsman et al., 2016). Additionally, this domain harbors a quality control activity (proofreading) to verify the correct configuration of the nascent peptide (Martín and Liras, 2017).

Intrinsic Product Modifying Domains

In addition to the C-domain related epimerization domain, discussed previously, there are cyclization (Cy), oxygenation (Oxy) as well as methyl-transferase (MT). These domains have been characterized to the extent of classifying their functions, although, especially Cy- and Oxy-domains may occur as a singular bi-functional unit or in a serial manner, respectively (Walsh, 2016). Cy- and Oxy-domains, specifically replace the classic function of C-domains, omitting amino acid condensation through peptide bond formation, resulting in thiazoline, oxazoline or methyloxazoline structures (Sundaram and Hertweck, 2016; Walsh, 2016). Those reactions predominantly occur in siderophore producing NRPS and rely on the presence of serine, threonine and cysteine residues (Patel et al., 2003; Kelly et al., 2005). Also MT-domains follow the common di-sub-domain structural patterning, which is also seen in A-, C-, E-, and Cy-domains. Fundamentally, MT-domains, however, are more restricted in their function, which covers the transfer of methyl-groups from *S*-adenosylmethionine to N (N-MT), C (C-MT), O (O-MT) or for certain residues S (S-MT) atoms resolving around the amino acids C_α carbon (Miller et al., 2003) and in case of S-MT C_β, respectively (Al-Mestarihi et al., 2014).

In *P. chrysogenum* 10 NRPS have been identified (**Table 1**) (van den Berg et al., 2008; Medema et al., 2011; Samol et al., 2016), of which only two have no attributed function. In this fungus, next to fungisporin (**Figure 1**) which is a cyclic hydrophobic tetrapeptide generated by a singular NRPS, three biosynthetic pathways involving a NRPS have been described in detail: penicillins, roquefortine/meleagrin, fungisporin and chrysogine (**Figure 4**).

Polyketides and Polyketide Synthase

Polyketides were already discovered in 1883 by James Collie, but the interest in these compounds (enzymes) was revived only as late as the 1950s by the work of Arthur Birch on the aromatic polyketide-6-methyl salicylic acid from *P. patulum*. These molecules are a class of NPs, that display different types of biological activities such as antibiotic (erythromycin A), antifungal (amphotericin B), immunosuppressant (rapamycin), antitumor (geldanmycin) and hypolipidemic (lovastatin) (Nair et al., 2012; Jenner, 2016; Weissman, 2016). Their assembly process is similar to that in the fatty acid biosynthesis, the main difference is the optional full reduction of the β-carbon in the PK biosynthesis. The group of enzymes that catalyzes the biosynthesis of PKs is referred to as PKSs (Keller et al., 2005; Caffrey, 2012).

In addition to the NRPSs, PKSs are the main enzymes that build the structural scaffold of a wide range of secondary metabolites and NPs in plants, bacteria, insects and fungi (Brakhage, 2012; Nair et al., 2012). Usually, these enzymes are encoded by genes that are grouped into clusters, that also specify genes encoding tailoring enzymes (oxygenases, oxidoreductases, reductases, dehydrogenases,and transferases), that further modify the scaffold produced by the PKS into a final product (Brakhage, 2012; Lim et al., 2012). PKSs are multimodular and multidomain enzymes that use a specific acyl-coenzyme A (acyl-CoA; usually malonyl-CoA or methylmalonyl-CoA) as building block, and subsequently catalyze a decarboxylative Claisen-type condensation of ketide units. The basic structural architecture consists of an acyl carrier protein (ACP), a ketosynthase (KS) and an acyltransferase (AT) domain. These combined domains extent a linear intermediate by two carbon atoms. An optional set of domains (dehydratase (DH), ketoreductase (KR), enoyl reductase (ER) and thioesterase (TE) may provide further modifications of the linear intermediate (Staunton and Weissman, 2001; Brakhage, 2012; Nair et al., 2012; Dutta et al., 2014).

According to their protein architecture and mode of action, PKS enzymes are classified into types I, II, and III (**Figure 5**). ***Type I PKSs*** are mainly found in bacteria and fungi. These multidomain proteins can be further subdivided in two categories: *modular* and *iterative* (Nair et al., 2012) *Modular type I PKSs* or *non-iterative PKSs* are unique for bacteria and are characterized by presenting a sequence (or set) of modules, each constituted with a set of specific catalytic domains. In consequence, the number of precursors fused in the PK is equivalent to the number of modules which are present (Chan et al., 2009). In contrast, *iterative type I PKSs* use the same catalytic core domains as modular type I PKSs, but the catalytic reaction is repeated to yield the complete PK backbone. A representative example of this type is LovB, that together with LovC (a enoyl reductase) catalyzes around 35 reactions to produce dihydromonacolin L, an intermediate in the lovastatin biosynthesis (Chan et al., 2009; Campbell and Vederas, 2010). Like iterative type I PKS enzymes, fungal PKSs (**Figure 2B**) are restricted to a single module and the consecutive domains act in sequential order during the synthesis of the complete PK. They are equipped with basic structural domains typically found in PKS enzymes (ACP-KS-AT domains) but may also contain optional units (KR, DH, ER, and Te domains). Depending on the presence or absence of reducing domains, these enzymes can be divided into *highly reducing (HR)*, *non-reducing (NR)* and *partially reducing (PR)* PKS (**Figure 2B**) (Keller et al., 2005; Crawford and Townsend, 2010; Jenner, 2016).

Highly Reducing PKS (HR-PKS)

Highly reducing PKS (HR-PKS) produce the linear or cyclic scaffold of some compounds such as fumonisins, T-toxins, solanapyrone E, squalestatin or/and lovastatin (Chiang et al.,

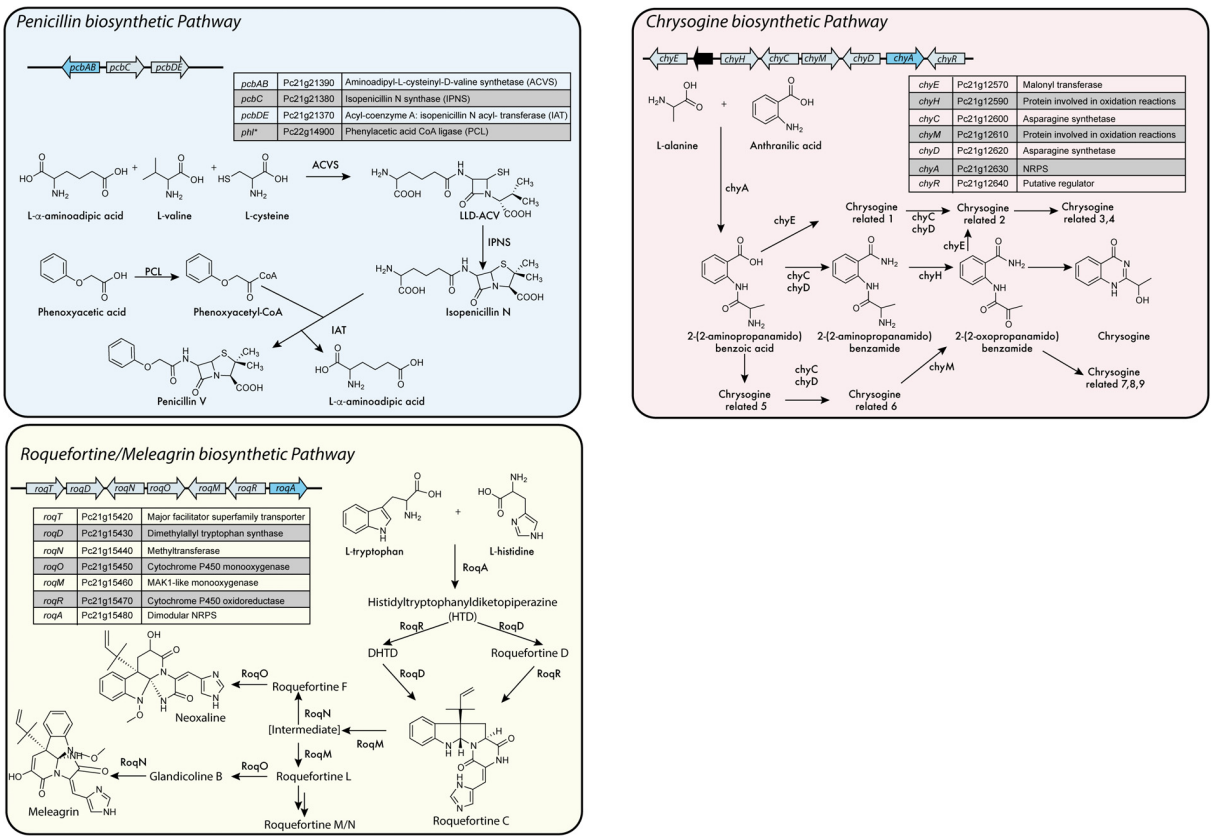

FIGURE 4 | NRPS based biosynthetic pathways in *P. chrysogenum*.*Is not part of the biosynthetic gene cluster (BGC). Adapted from Harris et al. (2009), Bartoszewska et al. (2011), García-Estrada et al. (2011), Weber et al. (2012c), Ali et al. (2013), Ozcengiz and Demain (2013), Ries et al. (2013), and Viggiano et al. (2017).

2014; Roberts et al., 2017). Usually, they start with a KS domain, followed by an AT, DH and C-Met domain, although the latter does not always follow the DH domain. The ER domain is an optional unit in HR-PKS enzymes, but when the ER is missing, the corresponding region is filled with a polypeptide domain with an unknown function. Furthermore, these enzymes do not contain a product template domain (PT) or N-terminal SAT domain, whereas these special domains are present in NR-PKS enzymes (Cox and Simpson, 2010).

Partially Reducing PKS (PR-PKS)

Structurally, these enzymes have a domain architecture that is similar to the mammalian FAS: a N-terminal KS-domain followed by an AT-, DH-, and "*core*"-KR-ACP domain. These

TABLE 1 | Nonribosomal peptide synthetases (NRPSs) in *P. chrysogenum* and known associated products.

Gene ID	Gene name	Protein	Domain organization	Product/Pathway
Pc13g05250	*pssC*	Siderophore synthetase	$A_1TCA_2TCTCA_3TCTCT$	Siderophore
Pc13g14330	–	Tetrapeptide synthetase	$CA_1TECA_2TCA_3TCA_3TCA_4TC$	–
Pc16g03850	*pssA**	Siderophore synthetase	ATCTA	Coprogen
Pc16g04690	*hcpA*	Cyclic tetrapeptide synthetase	$A_1TECA_2A_3TCA_4TECTCT$	Fungisporin
Pc21g01710	*nrpsA*	Dipeptide synthetase	A_1TCA_2T	Brevianamide F
Pc21g10790	–	Hexapeptide synthetase	$A_1TCA_2TCA_3TECA_4TCA_5TCA_6TC$	–
Pc21g12630	*chyA*	2-Aminobenzamide synthetase	A_1TCA_2TC	Chrysogine
Pc21g15480	*roqA*	Histidyl-tryptophanyldiketo- piperazine synthetase	A_1TCA_2TC	Roquefortine/Meleagrin
Pc21g21390	*pcbAB*	α-Aminoadipyl-cysteinyl-valine synthetase	$A_1TCA_2TCA_3TEte$	β-lactams
Pc22g20400	*pssB*	Siderophore synthetase	ATCTC	Fusarinines

*A, adenylation; T, thiolation; E, epimerization; te, thioesterase; C, condensation. *Point mutations present in nrps genes of industrial P. chrysogenum strains subject to CSI program. Modified from Salo et al. (2015), Samol et al. (2016), and Guzmán-Chávez et al. (2018).*

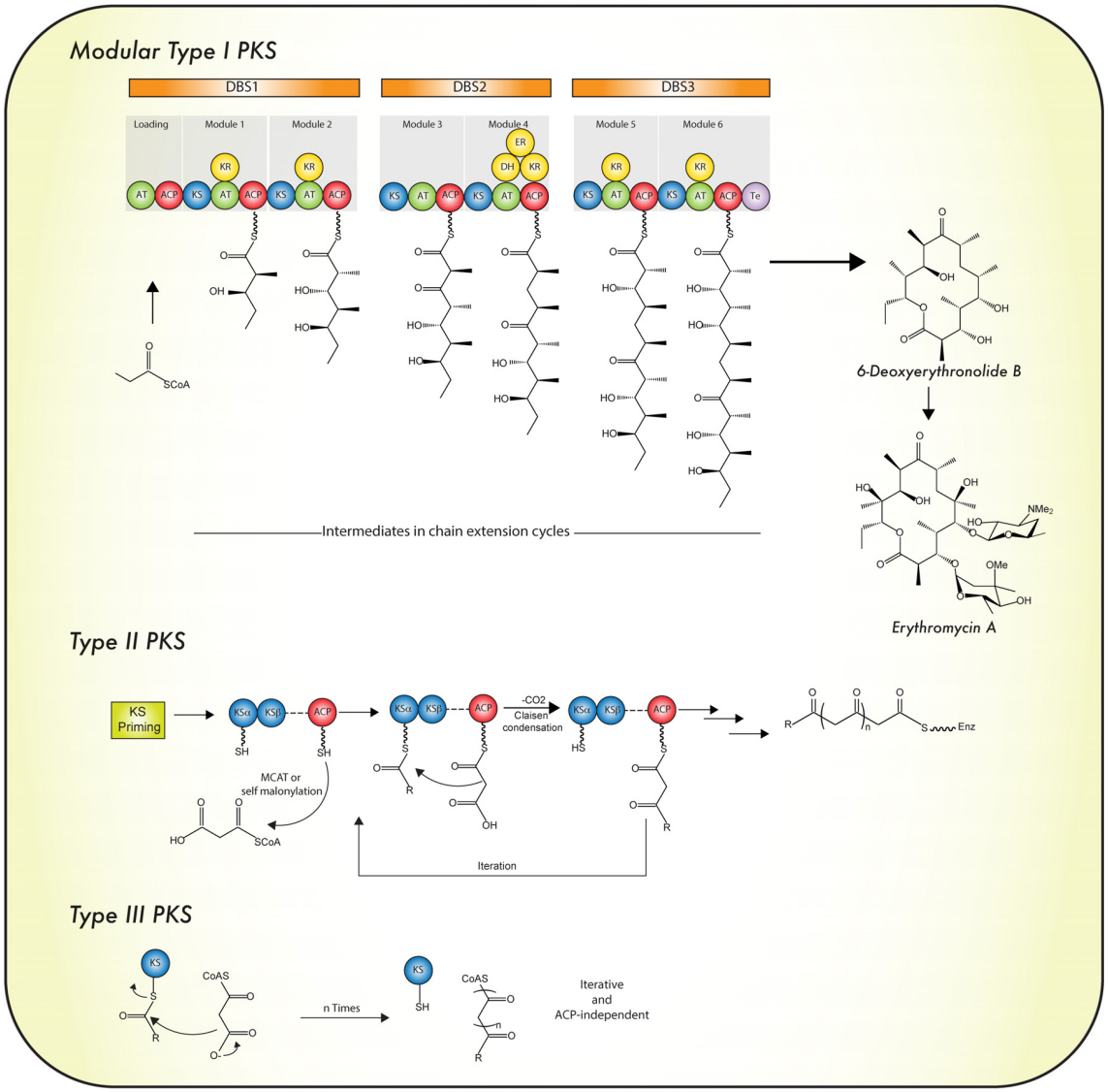

FIGURE 5 | Mode of biosynthesis of polyketide synthases. For details see the text. Abbreviations are as in the legend to **Figure 2**. Iterative type I PKS is depicted in **Figure 2B**.

enzymes lack an ER domain (Wang L. et al., 2015), and also do not have a Te domain, which suggests an alternative mechanism of product release than hydrolysis. PR-PKS enzymes produce small aromatic molecules such as 6-methylsalicylic acid (MSA), but in most cases the chemical product is unknown (Cox and Simpson, 2009, 2010; Kage et al., 2015).

Non-reducing PKS (NR-PKS; Aromatic PKs)

Non-reducing PKS (NR-PKS; aromatic PKs) typically, consist of six catalytic domains that are covalently associated and arranged in four components: *loading (SAT), chain extension (KS-MAT-PT-ACP), cyclisation and processing components (TE-CLC)* (Bruegger et al., 2013).

Type II PKSs are unique for bacteria and use a similar iterative mechanism as observed in *iterative type I PKSs*. However, the different catalytic domains are encoded by independent genes. In general, they often constitute a "minimal PKS," which comprises of two KS units (KS_α and KS_β) and an ACP protein that holds the growing PKS chain. The KS_β domain defines the length of the PK chain. The folding pattern of the poly-β-keto intermediates is determined by optional PKS units such as aromatases, ketoreductases, and cyclases. Other tailoring modifications are performed by oxygenases, methyl and glycosyl transferases. Known metabolites synthetized by type II PKSs are tetracyclines, anthracyclines and aureolic acids (Hertweck et al., 2007; Jenner, 2016). ***Type III PKSs*** have originally been discovered in plants but are also present in bacteria and fungi. They consist of a single KS domain that catalyzes a defined number of elongations, usually generating small phenols or naphtol rings. The enzyme transfers the acyl

group from the CoA to the active site histidine, which is a highly conserved residue. However, the amino acid sequence of the his motif is not similar to those found in KS domains of type I and II PKS enzymes (Shen, 2003; Chan et al., 2009; Bruegger et al., 2014; Jenner, 2016). Importantly, independent of the mechanistic or structural differences, all the PKs synthetized by PKS enzymes follow the same decarboxylative condensation mechanism of the acyl-CoA precursors. However, these precursors should be activated in prior by the ACP domain, in the case of the type I and II PKS enzymes, whereas type III PKS enzymes act independently of ACP domains (Shen, 2003; Hopwood, 2009). Acridones, pyrenes as well as (and) chalcones are some examples of the compounds produced by type III PKS enzymes (Yu et al., 2012). Below, a brief description is provided on the main catalytic features of PKS domains.

Acyltransferase Domains (AT)

A main unit during PK biosynthesis is the AT domain that selects the starter unit (malonyl-CoA or methylmalonyl-CoA) before it is transferred to the ACP domain for the chain elongation cycle (Dunn et al., 2013). This process involves two steps, i.e., the acylation and the transfer to the ACP (Jenner, 2016).

Acyl Carrier Protein (ACP)

The ACP is an essential cofactor that participates in PK biosynthesis. This protein belongs to a highly conserved carrier family, and consists of 70–100 amino acid residues (Byers and Gong, 2007). To perform the PK biosynthesis, the *holo-ACP* (active) form is generated by the phosphopantetheinyl transferase enzyme (PPTase) through a post-translational modification of ACP whereby a 4′-phosphopantetheine (4′-PP) moiety from CoA is transferred to the conserved serine (Evans et al., 2008; Kapur et al., 2010; Jenner, 2016) resulting in the formation of the Ppant arm. ACP modulates three important events during PK biosynthesis. First, it allows the condensation during chain elongations since it transfers the starter unit from the AT domain to the KS domain. Second, it shuttles the growing chain between the up and downstream domains, as well as to optional PKS domains, probably involving protein–protein recognition between domains. Third, it prevents premature cyclization and enolization of the PK chain (Yadav et al., 2013).

Ketosynthase Domains (KS)

The KS is a homodimeric condensing domain that catalyzes the extension of the β-ketoacyl intermediate by a decarboxylative Claisen condensation. This domain contains two active sites which are accessible to the ACP through its flexible Ppant arm, which receives the β-carboxyacyl-CoA extender unit from the AT. At that stage, a thioester bond is formed between the active-site cysteines' thiol group of the KS and the growing PK. Only when both units are covalently attached onto the module, a decarboxylative Claisen condensation occurs, which involves two conserved his residues. Therefore, mechanistically the KS domain acts at three stages: acylation, decarboxylation and condensation (Chen et al., 2006; Caffrey, 2012; Yadav et al., 2013; Jenner, 2016; Robbins et al., 2016).

Ketoreductase Domains (KR)

The KR domain functions as a β-carbon processing unit that belongs to the family of short-chain dehydrogenase/reductases. This domain reduces the β-keto group, that is formed during the condensation process, into a hydroxyl group (a β-hydroxyl intermediate) using NADPH (Keatinge-Clay and Stroud, 2006; Caffrey, 2012). Additionally, some KR domains are equipped with epimerase activity. The epimerizing module has a more open architecture, enabling the catalytic epimerization of methyl groups in acyl-ACP substrates, a reaction that involves the conserved serine and tyrosine residues which are also employed during ketoreduction (Ostrowski et al., 2016c; Bayly and Yadav, 2017).

Dehydratase Domains (DH)

The DH domain is usually coupled to B-type KR domains (B-type). This domain catalyzes water elimination (via *syn* or *anti*) at the β-hydroxy acyl chain position thereby producing *trans* double bonds (α,β-unsaturated moieties) (Caffrey, 2012; Bruegger et al., 2014; Jenner, 2016; Bayly and Yadav, 2017).

Enoyl Reductase Domains (ER)

The ER domain is an optional tailoring unit involved in the final oxidation state of the growing PK. It reduces α,β-enoyl groups and thereby generates saturated α–β bonds. This reaction involves NAD(P)H as hydride donor in a Michael addition type of mechanism. In the enoyl reduction, the products formed during this reaction have a specific stereochemistry (3R,2R) or (3R,2S) due to the β-carbon attack performed by the pro-4R hydride of NADPH, contrasting the KR domain that utilizes the pro-4S hydride (Chen et al., 2006; Bruegger et al., 2014).

Thioesterase Domain (Te)

Termination of PK biosynthesis involves the Te domain, which produces macrolactones via intramolecular cyclization or linear PKs by hydrolysis (Keatinge-Clay, 2012). In both events, an acyl-Te intermediate is formed through the transfer of the PK chain from the last ACP to the active serine on Te domain (Jenner, 2016).

Special Domains

In *non-reducing PKS,* the **ACP transacylase (SAT)** domain acts as starter unit that loads the ACP whereupon chain extension is mediated for KS and AT domain. During this process, the malonyl-CoA:ACP transacylase **(MAT) domain** transfers the extension units from malonyl-CoA to the ACP, while the product template **(PT) domain** stabilizes the reactive poly-β-keto intermediates. The processing component acts after the initial assembly when the cyclized or PK intermediate is still attached to the ACP. Final cyclization and release is catalyzed by the Te/Claisen cyclase **(CLC) domain** (Cox and Simpson, 2010; Crawford and Townsend, 2010; Bruegger et al., 2013; Chiang et al., 2014).

In *P. chrysogenum*, 20 PKS genes have been identified (**Table 2**) (van den Berg et al., 2008; Medema et al., 2011; Samol et al., 2016), but for only six the products are known. To date, in *P. chrysogenum* only four PK-related pathways have been

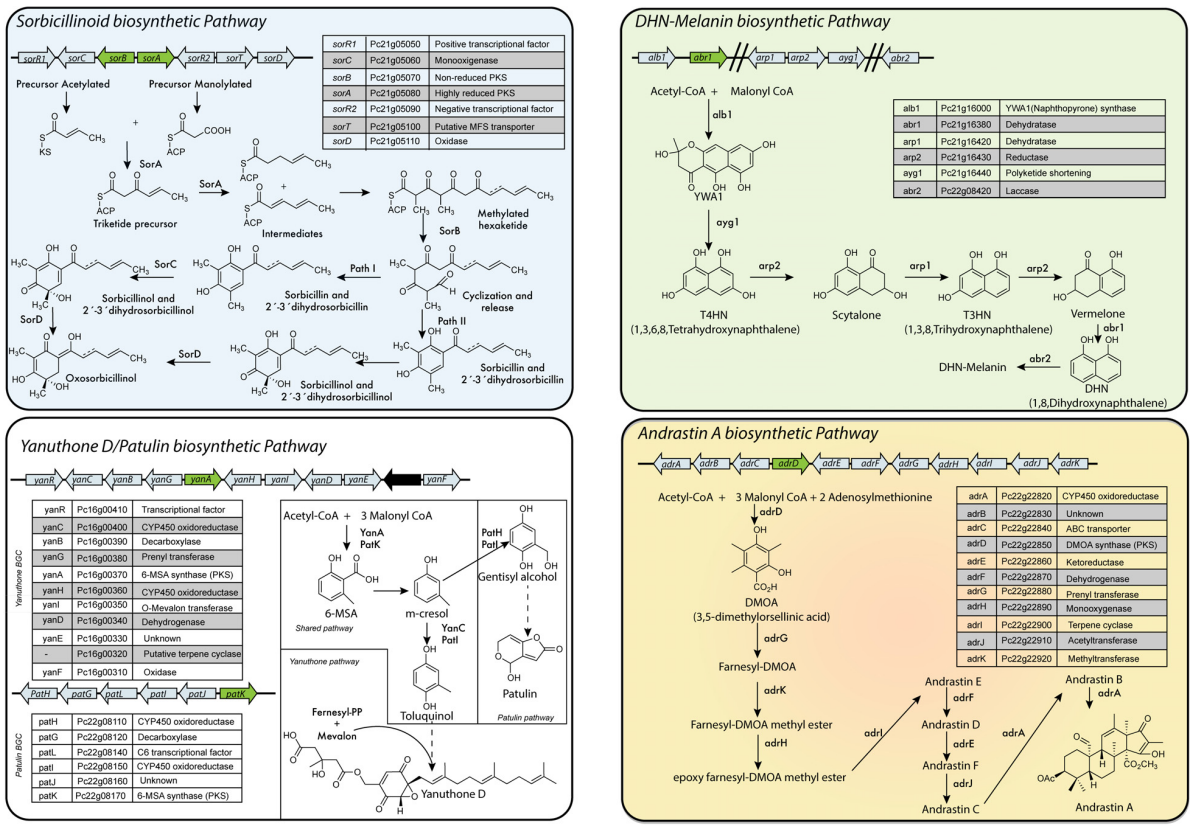

FIGURE 6 | PKS-based biosynthetic pathways in *P. chrysogenum*. *Sorbicillinoids:* Despite the fact that this cluster is also present in industrial strains of *P. chrysogenum,* they do not produce sorbicillinoids due to a point mutation in the ketosynthase domain of SorA. *Yanuthones/Patulin: P. chrysogenum* only contains a full version of one cluster (yanuthone D BGC), while the second cluster (patulin BGC) is incomplete (Nielsen et al., 2017). The absence of the gene encoding for an isoepoxidon dehydrogenase agrees with the fact that this fungi does not produce patulin (Samol et al., 2016). However, under laboratory conditions, yanuthone D is also not detected in this fungus (Salo, 2016). *DHN-Melanin:* The genes are only partially clustered in the genome of *P. chrysogenum*. *Andrastin A: P. chrysogenum* strains subjected to CSI are not able to produce andrastin A or related compound. Adapted from Staunton and Weissman (2001), Maskey et al. (2005), Cox (2007), Wattanachaisaereekul et al. (2007), Du et al. (2009), Pihet et al. (2009), Crawford and Townsend (2010), Avramovič (2011), Harned and Volp (2011), Gallo et al. (2013), Heinekamp et al. (2013), Matsuda et al. (2013), Salo et al. (2015), Salo et al. (2016), Druzhinina et al. (2016), Meng et al. (2016), Salo (2016), Samol et al. (2016), Guzmán-Chávez et al. (2017), Guzmán-Chávez et al. (2018), Nielsen et al. (2017), and Rojas-Aedo et al. (2017).

described in detail: sorbicillinoids, MSA-6/yanuthones, DHN-melanin and andrastin A (**Figure 6**).

Terpenoids Biosynthesis

In addition to NRPs and PKs, terpenoids are another class of NPs that are synthetized by filamentous fungi (Ascomycota) although less abundant as compared to Basidiomycota (Schmidt-Dannert, 2014). Fungal terpenoids or isoprenoids are structurally diverse molecules derived from isoprene units (C5 carbon skeleton): isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are synthetized in the mevalonate pathway from acetyl-CoA (Chiang et al., 2014; Soltani, 2016). The head-to-tail condensation of these C5 units is catalyzed by isoprenyl diphosphate synthases (IDSs), producing isoprenyl diphosphates with 10 (geranyl, GPP), 15 (farnesyl, FPP), and 20 (geranylgeranyl, GGPP) carbons. Eventually, these linear chains of different length are further modified by cyclases, terpene synthases (TPs) and prenyl transferases (PTs), yielding different subclasses of terpenoids (Schmidt-Dannert, 2014; Chen et al., 2016). For instance, monoterpenoids, sesquiterpenoids, diterpenoids, sesterterpenoids, and triterpenoids, which harbor two to six isoprene units, respectively (Soltani, 2016). Terpenoids are oxygenated derivatives of terpenes, which are also derived of isoprene (Stashenko and Martinez, 2017).

In filamentous fungi such as *Aspergillus*, *Penicillium, Claviceps*, and *Neosartorya*, ABBA-type PTs are involved in the biosynthesis of a range of toxins (Schmidt-Dannert, 2014). For the synthesis of indole-diterpenoids, IPPS-type PTs transfer GGPP to a indole group, while UbiA-type PTs are involved in the biosynthesis of meroterpenoids, which are hybrid NPs (terpenoids and PKs) (Itoh et al., 2010; Schmidt-Dannert, 2014). In *A. nidulans*, AusN (UbiA-type TPs) converts the product of a NR-PKS (3,5-dimethylorsellinic acid) as part of an earlier step in the dehydroaustinol/austinol biosynthesis pathway (Lo et al., 2012).

Terpene synthases catalyze cyclization reactions forming the carbocation by substrate ionization (class I) or substrate protonation (class II) (Zhou et al., 2012; Meguro et al., 2014).

A relevant example of class I TPs are sesquiterpene synthases, which cyclize the FPP to obtain a sesquiterpene scaffold (C15 backbone) (Quin et al., 2014). Recently, the *prx1* to *prx4* gene cluster involved in the biosynthesis of PR-toxin in *P. roqueforti* was cloned and sequenced. This cluster contains the gene *prx2* (*ari1*) that encodes for a aristolochene synthase which forms a sesquiterpene aristolochene derivative (precursor of PR-toxin). Interestingly, an orthologous gene cluster was identified in *P. chrysogenum* (Pc12g06300 to Pc12g06330), as part of BGC of eleven genes, which is also involved in the biosynthesis of PR-toxin (Hidalgo et al., 2014).

STRATEGIES FOR ACTIVATION OF BGCs

Natural products represent a broad range of molecules produced by animals, plants and microorganisms. These molecules may display different biological activities (e.g., antiviral, antimicrobial, anti-tumor, immunosuppressive agents) and it is estimated that the majority of these compounds are derived from filamentous fungal sources and from filamentous bacteria belonging to the genus *Streptomyces*. With respect to antibiotics, most of the chemical scaffolds used today were discovered during the golden age of antibiotics discovery (1940–1960s). This was followed by four decades during which hardly any new scaffolds from a natural source were developed (Reen et al., 2015; Smanski et al., 2016; Okada and Seyedsayamdost, 2017). However, there is also a current understanding that only a small fraction of the potential possible molecules has been discovered to this date. This follows from genomic studies revealing large numbers of uncharacterized BGCs, while many of these gene clusters are not expressed (silent or sleeping gene clusters) under laboratory conditions (Brakhage and Schroeckh, 2011). Furthermore, metagenomics studies indicate that the majority of microbes present in the environment have not been cultured nor characterized. Thus, there are many challenges that need to be overcome in order to harness the natural diversity of NPs, to cultivate potential strains under laboratory conditions and to activate the BGCs for expression. To achieve the synthesis of new NPs, three main approaches (**Figure 7**) were used in recent years, which may be successfully applied in *P. chrysogenum*: manipulation of cultivation conditions, engineering of NRPS and PKS and genetic interference.

Manipulation of Cultivation Conditions

Under natural conditions, fungi face a variety of biotic and abiotic conditions to survive. The cellular response to the environment involves complex regulatory networks that respond to stimuli such as light, pH, availability of carbon and nitrogen sources, reactive oxygen species, thermal stress, and interspecies-crosstalk (Brakhage, 2012; Reen et al., 2015).

OSMAC (One Strain Many Compounds) Approach

This strategy is derived from the observation that changes in the metabolic output of microorganisms can be achieved by alternating the medium composition and other cultivation parameters. It is well known that glucose, ammonium, or

TABLE 2 | Polyketide synthases in *P. chrysogenum* and (insofar known) their associated products.

Gene ID	Gene name	Protein	Domain organization	Product/Pathway
Pc12g05590	*pks1*	–	ks-at-dh-mt-kr-acp	–
Pc13g04470	*pks2**	–	ks-at-dh-mt-er-kr-acp	–
Pc13g08690	*pks3*	–	ks-at-dh-mt-er-kr-acp	–
Pc16g00370	*yanA*	6-MSA synthase	ks-at-kr-acp	6-MSA/Yanuthones
Pc16g03800	*pks5*	–	ks-at-dh-er-kr-acp	–
Pc16g04890	*pks6*	–	ks-at-dh-mt-er-kr-acp	–
Pc16g11480	*pks7**	–	ks-at-dh-mt-er-kr-acp	–
Pc21g00960	*pks8**	–	ks-at-dh-mt-er-kr-acp	–
Pc21g03930	*pks9*	–	ks-at-dh-mt-er-kr-acp	–
Pc21g03990	*pks10*	–	ks-at-dh-er-kr-acp	–
Pc21g04840	*pks11*	–	ks-at-dh-er-kr-acp	–
Pc21g05070	*sorB**	Sorbicillin synthase	ks-at-acp-mt-te/red	Sorbicillinoids
Pc21g05080	*sorA**	Sorbicillin synthase	ks-at-dh-mt-er-kr-acp	Sorbicillinoids
Pc21g12440	*pks14*	–	ks-at-dh-er-kr-acp	–
Pc21g12450	*pks15**	–	ks-at-acp-te	–
Pc21g15160	*pks16*	–	ks-at-dh-mt-er-kr-acp	–
Pc21g16000	*alb1**	YWA1 synthase	ks-at-acp-acp-te	YWA1/DHN-Melanin
Pc22g08170	*patK*	6-MSA synthase	ks-at-kr-acp	6-MSA
Pc22g22850	*adrD*	DMOA synthase	ks-at-acp-mt-te/red	DMOA/Andrastin A
Pc22g23750	*pks20*	–	ks-at-dh-mt-er-kr-acp	–

*ks, ketosynthase; at, acyltransferase; dh, dehydratase; mt, methyltransferase; er, enoyl reductase; kr, ketoreductase; acp, acyl carrier protein; te/red, thioester reductase. *Point mutations present in pks genes of industrial P. chrysogenum strains subject to CSI program. Modified from Salo et al. (2015), Samol et al. (2016), and Guzmán-Chávez et al. (2018).*

phosphate at high concentrations act as repressors of secondary metabolism, whereas iron starvation and nitrogen limitation can stimulate secondary metabolite production. The latter is for instance exploited for the production of terrain by *A. terreus* (Bode et al., 2002; Brakhage and Schroeckh, 2011; Gressler et al., 2015). This strategy can readily be implemented using high-throughput methods, where an array of culture conditions can be screened for new metabolite profiles (Spraker and Keller, 2014). In combination with bioinformatics tools, this strategy can be a powerful tool to investigate the production of new molecules, as exemplified by the discovery of aspoquinolones A–D in *A. nidulans* (Scherlach and Hertweck, 2006). However, despite the fact that the OSMAC approach has led to the discovery of increased numbers of new molecules with antimicrobial activity, some chemical and physical conditions are still missing under the laboratory tested conditions as the activation often concerns a limited number of BGCs (Chiang et al., 2009).

Interspecies-Crosstalk

The production of secondary metabolites is a natural strategy that microorganisms have developed to cope with specific environmental conditions and challenges. They serve as intermediary agents to establish a symbiotic association between species or as a weapon against other organism to compete for nutrients and space. These conditions, that are not present in axenic cultures, boost the production of molecules that are constitutively present and/or that are cryptic and normally are not synthetized due to silencing of the respective BGCs (Demain and Fang, 2000; Marmann et al., 2014). The strategy in which different organisms are cultivated together is called "*co-culture,*" which has been successful in several cases yielding new metabolites. *A. fumigatus* produces fumiformamide when co-cultivated with *Streptomyces peucetius*, while co-cultivation of this fungi with *S. rapamycinicus* results in the production of fumicyclines A and B, two novel PKs with antibacterial activity, are examples of the use of this strategy (Netzker et al., 2015; Adnani et al., 2017). Interestingly, the association of two marine organisms, *Emericella* sp. and *Salinispora arenicola*, results in the biosynthesis of emericellamides A and B which are equipped with antibacterial activity (Oh et al., 2007). Also, the interactions between fungi and insects result in the production of volatile secondary metabolites (Rohlfs and Churchill, 2011).

Engineering of NRPS and PKS

Nonribosomal peptide synthetase and PKS are highly structured and multi-facetted enzymes, containing a tremendous potential for the exploitation of their product scaffold structure for the generation of novel, bioactive compounds. However, due to the complexity of all interactions within these mega enzymes, the elucidation and implementation of engineering strategies is an extremely challenging task. Several strategies have been developed and applied with different degrees of success, though the overall approaches can be grouped as module, domain, sub-domain or site directed, respectively. Owing to their large size, utilization of a random mutagenesis approach proved to be difficult, but other more directed strategies are met with a great success. Nevertheless, all of these strategies have their inherent difficulties, advantages and disadvantages in respect to the complexity and success rate of NRPS/PKS engineering efforts.

Subunit, Module, and Domain Swapping

Extensive efforts targeting the active site of A-domains has been a major focus in NRPS engineering. Multiple studies confirmed that the substrate specificity of a NRPS A-domain can be successfully altered, however, at the cost of substantially lowered catalytic velocity (Thirlway et al., 2012; Zhang et al., 2013). Similar successes and limitations were observed when domains were swapped or replaced by synthetic versions (Beer et al., 2014). The most challenging way of obtaining novel NRPS, however, is the swapping or combining of entire modules (Kries, 2016). Domain swapping overall created not only functional parts or domains, but also complete NRPS though with limited success (Beer et al., 2014).

Due to the strict arrangement of NRPS in domains and modules, the possibility of exchanging a unit appears to be the most straight forward approach for altering its intrinsic properties. A series of studies targeting the enzymes linked to the production of daptomycin (Nguyen et al., 2006; Baltz, 2014) elucidated the possibilities and borders of a combinatorial swapping strategy in context of novel compound production. The daptomycin biosynthetic cluster comprises three NRPS containing a total of 13 modules for the incorporation of an equal number of substrates. Different levels of domain and module swap approaches were followed, starting with the exchange of modules 8 and 11 (C-A-T), representing an internal module exchange. The resulting NRPS exhibited the production of novel daptomycin compounds with an inverted amino acid composition at the predicted sites at a near native rate (Nguyen et al., 2006). A similar combinatorial approach has been chosen for altering the PK stereochemistry. The exchange of a R domain with a TE domain in a NR-PKS from *A. niger* produced two alternative NR-PKS that harbor carboxylic acids instead of the aldehydes present in the original products (Yeh et al., 2013; Weissman, 2016). In *Aspergillus*, this rational domain swap has also been used to diversify the native substrates that NR-PKS takes as starter unit to produce new products. This involved exchanging the starter unit ACP transacylase domain in the PKS (Liu et al., 2014). Likewise, an analogous approach was used to produce new hybrids (PK–NRPs) in *A. nidulans* via module swapping of the two PKS–NRPS natural hybrids involved in the syn2 and cytochalasin E pathways from *Magnaporthe oryzae* and *A. clavatus* respectively (Nielsen et al., 2016). Despite the successful use of this strategy in some filamentous fungi, the engineering of NRPS and PKS in *P. chrysogenum* remains unexplored.

Genetic Interference

Another mechanism to stimulate the expression of silent BGCs in *P. chrysogenum* is by genetic interference, for instance by direct manipulation of the regulatory network related to BGCs expression. The regulation of BGCs is effected at many levels, through specific (or local) and global regulators up to epigenetic

regulation involving the modification of the chromatin landscape (Lim et al., 2012; Spraker and Keller, 2014).

Global and Specific Regulators

Global regulator-based regulation

Pleiotropic transcriptional regulators or global regulators are proteins that respond to environmental signals such as pH, temperature, and N- and C-sources. They provide the link between the production of secondary metabolites and external cues. In fungi, these proteins control the regulation of BGCs that do not contain other regulatory factors. Up to 40% of the known clusters do not encode a local and specific regulator (or obvious regulatory genes). Additionally, global regulators also act on genes that do not belong to secondary metabolism (Brakhage, 2012; Rutledge and Challis, 2015; Fischer et al., 2016). Global regulators that have been reported as key players in the biosynthesis of secondary metabolites are featured below.

Velvet complex. This heterotrimeric complex is a conserved regulator present in most of the fungi, except yeast. It consists of at least three proteins: VeA, VelB, and LaeA. Likewise, this complex provides a link between sexual development and secondary metabolism through light regulation (Yin and Keller, 2011; Deepika et al., 2016), since light has an inhibitory effect on VeA expression. The formation of the velvet complex takes place in the nucleus, where the complex VeA–VelB via the α-importin KapA meets the methyltransferase LaeA. It has been hypothesized that the velvet complex acts as a transcriptional factor as it contains a DNA binding fold that resembles the corresponding region of the NF-κB transcription factor of mammals (Sarikaya-Bayram et al., 2015). The role of the velvet complex in secondary metabolism mostly follows from the control that the LaeA protein executes on several BGCs in filamentous fungi. LaeA (loss of aflR expression-A) was identified in 2004 as a global regulator in *Aspergillus*. Deletion of this gene results in the repression of many BGC, such as the one responsible for the production of penicillin, lovastatin, and sterigmatocystin. Overexpression of LaeA causes an opposite phenotype. Interestingly, LaeA is negatively regulated by AflR (Zn_2Cy_6 transcriptional factor) in a feed loop mechanism (Bok and Keller, 2004). It has been hypothesized that LaeA acts at different levels, i.e., as a methyltransferase, epigenetically and as a direct member of the velvex complex. Structurally, LaeA has a *S*-adenosyl methionine (SAM)-binding site with a novel *S*-methylmethionine auto-methylation activity, although this activity does not seem to be essential for its function. LaeA is not a DNA-binding protein, but it does affect chromatin modifications. In an *A. nidulans* Δ*laeA* strain, high levels of the heterochromatin protein 1 (HepA) are detected and an increase in trimethylation of the H3K9 in the sterigmatocystin cluster. When LaeA is present, the levels of HepA, ClrD (H3K9 methyltransferase) and H3K9me3 decrease while the sterigmatocystin levels are raised. The heterochromatic marks stay until the sterigmatocystin cluster is activated, and apparently LaeA influences the offset of these marks in this particular cluster (Reyes-Dominguez et al., 2010; Brakhage, 2012; Jain and Keller, 2013; Sarikaya-Bayram et al., 2015; Bok and Keller, 2016). Orthologs of LaeA have been discovered in many other filamentous fungi as *Penicillium*, *Fusarium*, *Trichoderma*, *Monascus* spp. and LaeA exhibits positive and negative effects on the synthesis of NPs. For instance, LaeA1 of *F. fujikuroi* positively regulates the production of fusarin C, fumonisins and gibberellins, and represses bikaverin biosynthesis. In *P. chrysogenum,* LaeA controls the biosynthesis of penicillin, pigmentation and sporulation (Keller et al., 2005; Kosalková et al., 2009; Jain and Keller, 2013). In *Trichoderma reesei*, Lae1 positively modulates the expression of cellulases, xylanases, β-glucosidases. Interestingly the stimulation of these genes was not directly influenced by the methylation of H3K4 or H3K9 (Wiemann et al., 2010; Yin and Keller, 2011; Lim et al., 2012; Seiboth et al., 2012; Jain and Keller, 2013).

LaeA is not the only member of the velvet complex that has influence on the regulation of secondary metabolite production. *VeA* of *A. parasiticus* is necessary for the expression of two transcriptional factors of the aflatoxin cluster (AflR and AflJ), which regulate the pathway. In *A. fumigatus*, veA regulates 12e BGCs (Dhingra et al., 2013). This study also revealed that veA modulates the biosynthesis of fumagillin via the regulation of FumR, a transcriptional factor of the fumagillin cluster, which in turn is also regulated by LaeA. Similarly, a transcriptome analysis in *A. flavus* revealed that 28 of 56 BGCs are dependent on *veA,* in particular the aflavarin cluster which is differentially expressed. Likewise, orthologs of veA are also present in other fungi such as in *P. chrysogenum*, *F. oxysporum, Botrytis cinerea*, *F. verticillioides* (Yin and Keller, 2011; Dhingra et al., 2013; Jain and Keller, 2013; Cary et al., 2015). Despite the clear interaction between veA and LaeA in the velvet complex and its influence on secondary metabolism, it is thought that veA may be acting as molecular scaffold of the velvet complex, since it interacts also with three other methyl transferases [LaeA-like methyltransferase F (LlmF), velvet interacting protein C (VipC), and VipC associated protein B (VapB)]. This suggests that veA functions in a supercomplex or in dynamic network control. Taken together, modulation of the velvet complex is a useful tool to activate BGCs (Sarikaya-Bayram et al., 2015) but results are difficult to predict.

bZIP transcription factors. Basic leucine zipper (bZIP) transcription factors are highly conserved in the eukaryotes. The dimeric bZIP transcriptional factors play an important role in the cellular responses to the environment. Regarding their structure, they contain a conserved leucine zipper domain and a basic region, which controls the dimerization of the protein and establishes sequence-specific DNA-binding, respectively. Once dimeric, bZIPs target palindromic DNA sequences by two mechanisms: redox and phosphorylation (Amoutzias et al., 2006; Knox and Keller, 2015). In fungi, bZIP proteins have been implicated in multiple metabolic processes, such as in the regulation of development, morphology and in stress responses. Several orthologs of the Yap family bZIPs, which were first described in yeast, have been characterized in *Aspergillus* spp. (AtfA, NapA, Afyap1, Aoyap1, and Apyap1) and these regulators have recently been associated with the production of secondary metabolites in filamentous fungi. In *A. nidulans*, overexpression of *RsmA* (restorer of the secondary metabolism A, Yap-like bZIP) has a compensatory effect on secondary metabolism in a strain in

which LaeA and veA are missing. However, these transcription factors also display negative regulation. For instance, an increase in the biosynthesis of aflatoxin and chratoxin has been observed when *yap1* is deleted in *A. parasiticus* and *A. ochraceus* (Yin et al., 2013; Knox and Keller, 2015; Wang X. et al., 2015). MeaB is another bZIP transcriptional factor which was discovered in *A. nidulans*. Its function is associated in nitrogen regulation and has a negative effect on the biosynthesis of aflatoxin in *A. flavus* and bikaverin production in *F. fujikuroi* (Wagner et al., 2010; Amaike et al., 2013).

Other global regulators. AreA is a highly conserved transcriptional factor in fungi that belongs to the GATA family and it is characterized by Cys_2His_2 zinc finger DNA binding domains. Likewise, it is involved in the repression of nitrogen metabolism when ammonium or glutamine are present. Recently, this transcription factor and its orthologs have been shown to influence secondary metabolism. For instance, *areA* deletion strains of *F. verticillioides* are not able to produce fumonisins on mature maize kernels. In *Acremonium chrysogenum*, the deletion of *areA* resulted in the reduction of cephalosporin because of a reduced expression of the enzymes involved in cephalosporin biosynthesis. Additionally, AreA is a positive regulator of the production of gibberellins, trichothecene deoxynivalenol (DON), fusarielin H, beauvericin and zearalenone (Li et al., 2013; Tudzynski, 2014; Knox and Keller, 2015 and Keller, 2015). The carbon catalytic repressor CreA also influences secondary metabolism. CreA is a Cys_2His_2 zinc finger transcription factor that is involved in the repression of genes associated with the use of carbon sources other than glucose (Knox and Keller, 2015). This transcription factor acts by direct competition with activator proteins for specific binding sites (5′-SYGGRG-3′) and by direct interaction with activators (Janus et al., 2008). In *P. chrysogenum* CreA represses penicillin biosynthesis and causes a reduced expression of the *pcbAB* gene that encodes a NRPS involved in this pathway. Mutations in the putative CreA binding site in the *pcbAB* promoter result in enhanced enzyme expression when cells are grown in the presence of glucose (Cepeda-García et al., 2014). In contrast, mutations in the CreA binding sites of the *ipnA* promoter (*pcbC* in other species) of *A. nidulans* revealed that in this organism repression of penicillin biosynthesis by glucose is independent of CreA (Knox and Keller, 2015). CreA has been implicated in the variable metabolite profiles when fungi are grown in the presence of different carbon sources (Yu and Keller, 2005). Recently, the xylanase promoter binding protein (Xpp1) of *Trichoderma reesei* was used as a reporter to fulfill a dual role in the regulation of primary and secondary metabolism. Xpp1 is an activator of primary metabolism, while its deletion boosts the production of secondary metabolites, including sorbicillinoids (Derntl et al., 2017b). Another Cys_2His_2 zinc finger transcription factor conserved in fungi is PacC, which is involved in pH dependent regulation. Deletion of the ortholog of this gene (BbpacC) in *Beauveria bassiana* resulted in a loss of dipicolinic acid (insecticide compound) and oxalic acid production, compounds that reduce the pH of the medium. However, also production of a yellow pigment was noted. When *A. nidulans* is grown at alkaline pH, PacC modulates the expression of the *acvA (pcbAB)* and *ipnA* of the penicillin BGC, while it acts negatively on the expression of the sterigmatocystin BGC (Deepika et al., 2016; Luo et al., 2017). In filamentous fungi, another global regulatory element is the CCAAT-binding complex (CBC). This complex consists of three proteins (HapB, HapC, and HapE) that respond to redox stimuli and an additional unit HapX, a bZIP protein that interacts with the complex for modulating the iron levels. In *A. nidulans* this complex binds to CAATT motifs, which are present in the penicillin BGC stimulating the expression of the *ipnA* and *aatA* (*penDE*) genes (Bayram and Braus, 2012; Brakhage, 2012). Whereas in *F. verticillioides* the ortholog core of this complex (FvHAP2, FvHAP3, and FvHAP5) is deleted, cells show an altered hyphal morphology, reduction of growth, reduced pathogenesis and a deregulation of secondary metabolism (Ridenour and Bluhm, 2014).

Specific regulator-based regulation

In addition to the global regulators, the expression of BGCs can be also modulated by specific regulatory elements, which most of the times are encoded by genes that are part of the same cluster that they regulate. In some cases, such regulators also influence the expression of other BGCs. It is estimated that about 60% of the fungal BGCs contain a gene encoding a potential regulator amidst the gene cluster. With PKS containing BGCs mostly containing a regulator that belongs to the Zn_2Cys_6 binuclear cluster domain family (around 90%). With NRPS containing BGCs, the putative transcription factors are more diverse. The Zn_2Cys_6 family of transcription factors contain a DNA binding domain (DBD) that has two zinc atoms coordinated by six cysteines. There are three sub regions: a linker, a zinc finger and a dimerization domain. Additional to a DBD, these proteins contain two further functional domains, the acidic region and the regulatory domain. These transcription factors can act as monomers, hetero- and homodimers. They recognize single or multiple trinucleotide sequences, commonly CCG triples, in a symmetric or asymmetrical format. The affinity of the DBD for a given DNA stretch is also determined by the nucleotides surrounding this triplet. The transcriptional activity of these proteins is regulated by phosphorylation, exposing the activation and DNA binding domains for DNA binding (MacPherson et al., 2006; Brakhage, 2012). Some of these regulators have been shown to control the expression of BGCs. For instance, in *F. verticillioides* the disruption of *FUM21* gene, that encodes a Zn_2Cys_6 protein, reduces fumonisin production as a result of a downregulation of the BGC (Brown et al., 2007). Interestingly, fumonisin production is also regulated by another Zn_2Cys_6 protein that is encoded by a gene located outside of the fumonisin cluster (Flaherty and Woloshuk, 2004). Mlcr is another example of a positive regulator that controls compactin production in *P. citrum* (Abe et al., 2002). AflR is a Zn_2Cys_6 protein that regulates the biosynthesis of aflatoxin/sterigmatocystin through binding to a palindromic sequence (5′-TCG(N_5)GCA) that is found in most of the promoters of this BGC, albeit a second binding sequence has been reported that is associated with the autoregulation mechanism of the expression of *AflR*. The disruption of *AlfR* abolishes

the production of aflatoxin/sterigmatocystin. Likewise, some BGCs encode multiple regulatory proteins. Next to the *aflR* gene in the aflatoxin cluster resides the *aflS* (formerly *aflJ*) gene. The corresponding transcription factor binds to AflR to enhance the transcription of early and mid-biosynthetic genes in the aflatoxin pathway (Georgianna and Payne, 2009; Yin and Keller, 2011). In *P. chrysogenum* and *Trichoderma reesei*, the sorbicillin BGC is regulated by two transcriptional factors through a coordinated action (Derntl et al., 2016, 2017a; Guzmán-Chávez et al., 2017). Also, regulation of BGCs via crosstalk has been observed in filamentous fungi. For instance, the alcohol dehydrogenase promoter has been used to induce the expression of putative pathway-specific regulatory gene *(scpR)* in *A. nidulans*, which controls the expression of two pathway associated NRPS genes (*inpA* and *inpB*). Surprisingly, two PKS genes (*afoE* and *afoG*) and one transcriptional activator (*afoA*) belonging to the asperfuranone BGC are also upregulated by ScpR, allowing the production of asperfuranone (Bergmann et al., 2010). For some regulators, no clear phenotype is observed. For instance deletion of the *chyR* gene of the chrysogine BGC in *P. chrysogenum*, has no effect on the expression of the corresponding BGC (Viggiano et al., 2017).

Manipulation of regulatory elements as strategies for the activation of BGCs

Gene deletion. It is a classical strategy that consists of the abolishment of the expression of a certain gene by its elimination whereupon the impact on the metabolite profile is examined by HPLC or LC-MS. A major limitation of this approach is that it can only be used in BGCs that are not totally silenced under laboratory conditions. Using this strategy, it was possible to elucidate the highly branched biosynthetic pathway for the synthesis of roquefortine as well as the biosynthetic pathways of sorbicillinoids and chrysogine in *P. chrysogenum* (García-Estrada et al., 2011; Ali et al., 2013; Ries et al., 2013; Deepika et al., 2016; Guzmán-Chávez et al., 2017; Viggiano et al., 2017). Likewise, this approach can be used to remove transcriptional repressor genes, as in the case of TetR-like pathway-specific repressor proteins, whose deletion induced the production of gaburedins in *Streptomyces venezuelae* (Rutledge and Challis, 2015). Global regulators, such as LaeA have also been targeted using this strategy (Chiang et al., 2009).

Promoter replacement. Another method concerns the replacement of the endogenous promoter of the gene(s) in a BGC by a strong constitutive or inducible promoter. For instance in *A. nidulans* replacement of the native promotor of the *scpR* gene (secondary metabolism cross-pathway regulator) for the inducible promoter of alcohol dehydrogenase AlcA induced the expression of a silent cluster that contained two NRPS genes (*inpA* and *inpB)* and *scpR* itself. Additionally, it also led to the expression of the asperfuranone BGC, which is normally silent (Bergmann et al., 2010; Yin and Keller, 2011; Lim et al., 2012). Recently in *P. chrysogenum* a promising promoter toolbox for bioengineering purposes was developed. This included the analysis of four constitutive promoters from *P. chrysogenum* and six from *A. niger*, which were evaluated using a reporter system and assorted by promoter strength (Polli et al., 2016).

Overexpression of a specific or global regulator. This approach is one of the most used strategies to turn on cryptic BGCs, since a change in expression level of a regulator may boost the expression of a whole cluster. Usually, this strategy is applied in combination with the *promoter replacement* approach. Using this strategy, i.e., overexpression of the transcription activator ApdR under control of the alcohol dehydrogenase promoter *alcAp*, it has been possible to induce the expression of a hybrid PKS-NRPS BGC in *A. nidulans*. This resulted in the production of aspyridones A and B (Bergmann et al., 2007). Similarly when the global regulator FfSge, which is associated with vegetative growth of *F. fujikuroi*, is overexpressed, some BGCs are forced to express under these unfavorable conditions (low nitrogen concentrations) leading to the identification of the corresponding products (Michielse et al., 2015).

Chromatin-Mediated Regulation

In fungal cells, chromosomal DNA is wrapped in a complex of DNA, histone proteins and RNA called chromatin. This chromatin structure consists of a basic unit called nucleosome, which consists of superhelical DNA (147 base pairs) that binds an octamer of four different core histone proteins (two each of H2A, H2B, H3, and H4) in 1.75 turns. It has been shown that modifications of the chromatin structure (boosts or alters) changes gene expression, amongst other genes involved in the biosynthesis of secondary metabolites. Structurally, chromatin represents an obstacle that complicates access of DNA-binding factors to their corresponding binding regions. According to the compaction level, chromatin can be in a dense (heterochromatin) or relaxed (euchromatin) state. These compaction levels are regulated by post-translational modification of the histone proteins by acetylation, methylation, ubiquitination, ethylation, propylation, butylation, and phosphorylation events. Regions that display low transcriptional activity have been associated with the heterochromatic conformation. In contrast, the euchromatic conformation is present in regions with abundant coding sequences and is usually highly active during transcription. Such regions are also linked with hyper-acetylated nucleosomal histones. Likewise, it has been reported that methylation of H3K9, H3K27, and H4K20 are typical markers of the heterochromatin, while in euchromatin methylation occurs at H3K4 (Brosch et al., 2008; Strauss and Reyes-Dominguez, 2011; Gacek and Strauss, 2012; Spraker and Keller, 2014; Rutledge and Challis, 2015).

Histone methylation, acetylation, and sumoylation

As mentioned above, LaeA influences secondary metabolite production through chromatin modification. The methylation state of H3K9 has been correlated with the heterochromatin protein A (HepA), since this protein needs the di- and tri-methylation of H3K9 for binding to chromatin and to form heterochromatin. Deletion of LaeA allows the unobstructed binding of HepA to the *AlfR* promoter, thereby affecting the expression of the sterigmatocystin pathway. The deletion of the methyltransferase encoding *clrD* and *ezhB* genes in *Epichloe festucae,* that act on H3K9 and H3K27, respectively (in axenic culture), results in the activation of the ergot alkaloids and lolitrem BGCs. These compounds are necessary to establish a

symbiotic association with the plant *Lolium perenne*. Compass (complex of proteins associated with Set1) which methylates H3K4 in yeast, also impacts secondary metabolism in filamentous fungi. The deletion of one of its components (*cclA*) in *A. nidulans* allowed the activation of a cryptic BGC and the production of emodin (Palmer and Keller, 2010; Gacek and Strauss, 2012; Chujo and Scott, 2014; Netzker et al., 2015; Deepika et al., 2016). Likewise, in *F. fujikuroi* and *F. graminearum,* the deletion of *cclA* caused the overproduction of secondary metabolites derived from BGCs close to the telomeres, but this seems to relate to a H3K4 methylation independent mechanism (Studt et al., 2017). Other types of histone modification may alter the chromatin landscape, such as acetylation which is a reversible process governed by two antagonist enzymes: histone acetyltransferases (HATs) and deacetylases (HDACs). Active transcription is usually associated with histone acetylation, although recently the deacetylation of histones has been shown to cause activation of genes (Brosch et al., 2008). Usually, histones are acetylated by several complexes with acyltransferase activity, such as Saga/Ada and NuA4. In *A. nidulans* a chromatin immunoprecipitation (ChIP) analysis revealed that GcnE and AdaB, the catalytic subunits of the complex Saga/Ada, are needed for acetylation of histone H3 (Deepika et al., 2016). Indeed, the interaction between *A. nidulans* and *Streptomyces rapamycinicus* can be linked to a GcnE dependent increase in the acetylation of H3K14 that shields the promoters of the orsellinic acid BGC. The Saga/Ada complex is a key player in the induction of the penicillin, terrequinone and sterigmatocystin BGCs (Nutzmann et al., 2011; Brakhage, 2012). In contrast, deletion of *hdaA* (encoding a HDAC) in *A. nidulans* resulted in major changes in the metabolite profile (Rutledge and Challis, 2015). HdaA is a class 2 histone deacetylase involved in the regulation of BGCs that are located near the telomeres, such as the penicillin and sterigmatocystin clusters in *A. nidulans*. Indeed, deletion of the *hdaA* gene results in the increased and early gene expression of these two BGCs, and the production of the corresponding secondary metabolites. In *A. fumigatus*, the *hdaA* gene is involved in growth and production of secondary metabolites, and the deletion of this gene increases the production of many secondary metabolites while it causes a reduction of gliotoxin production. In contrast, HdaA overexpression shows the opposite effect (Shwab et al., 2007; Lee et al., 2009). In *P. chrysogenum* was demonstrated that HdaA (histone deacetylase) mediates the transcriptional crosstalk among sorbicillinoids biosynthesis and other BGCs, since a new compound as detected only under conditions of sorbicillinoids production (Guzmán-Chávez et al., 2018).

Histone deacetylases are ubiquitously distributed in filamentous fungi, and therefore HDAC inhibitors can be used to improve the synthesis of NPs by epigenome manipulation (Shwab et al., 2007; Lee et al., 2009). For instance, the metabolite profile of *Cladosporium cladosporioides* and *A. niger* underwent a significant change when these strains were exposed to suberoylanilide hydroxamic acid (SAHA), a HDAC inhibitor, allowing the detection of two new compounds, cladochrome and nygerone A, respectively (Rutledge and Challis, 2015). An exploratory analysis performed in 12 fungi treated with different types of DNA methyltransferase and histone deacetylase inhibitors, revealed the production of new secondary metabolites but also the elevated amounts of known compounds (Williams et al., 2008). In this respect, the chromatin state can directly influence the binding of transcription factors, and thereby modulate expression (Palmer and Keller, 2010; Macheleidt et al., 2016). It has been hypothesized that histone sumoylation may modulate secondary metabolite production. This process is mediated by a small protein termed SUMO (small ubiquitin-like modifier) that shares structural similarity to the ubiquitin protein. In *A. nidulans*, deletion of the *sumO* gene enhanced the production of asperthecin, whereas synthesis of austinol, dehydroaustinol, and sterigmatocystin was reduced. Although the molecular mechanism still needs to be elucidated, it is thought that sumoylation acts at several levels, such as on epigenetic regulators (COMPASS, Clr4, SAGA/ADA and HDACs) or at the level of transcriptional regulators (Brakhage and Schroeckh, 2011; Spraker and Keller, 2014; Wu and Yu, 2015).

Modification of the chromatin landscape to activate BGCs

Many fungal BGCs are located in distal regions of the chromosomes. In these heterochromatin rich regions, transcription of the BGCs can be activated by epigenetic regulation. Therefore, the encoding genes of proteins that influence histone modification are prime targets, although these modifications can also be achieved by chemical treatment (Williams et al., 2008; Brakhage, 2012). A recent study in *P. chrysogenum* showed that the expression of a set of PKS and NRPS encoding genes is induced when an ortholog of a class 2 histone deacetylase (HdaA) is deleted. This allowed for the overproduction of sorbicillinoids, the reduction of chrysogine related metabolites and the detection of a new compound whose origin still unknown (Guzmán-Chávez et al., 2018).

Other Targets for Regulation

Secondary metabolites produced by fungi can be toxic to the producer organisms, and often fungi are equipped with detoxification mechanisms. One of these mechanisms is toxin excretion by transporters, which are membrane proteins whose genes often localize to the BGCs. Transporters may belong to different protein families but the major facilitator superfamily (MFS) and ABC superfamily are most commonly encoded by BGCs (Keller, 2015). Since biosynthesis of secondary metabolites may take place in different cell compartments, also intracellular transport may be evident (Kistler and Broz, 2015). Despite their assumed biological importance, the deletion of transporter genes from the BGCs often does not impact secondary metabolite production. For instance, deletion of the *A. parasiticus aflT* gene, that encodes a MFS transporter, does not result in reduced aflatoxin excretion, despite the fact that *aflT* belongs to the aflatoxin BGC and its expression is regulated by a specific transcription factor, AflR, of the pathway. Probably, this protein is redundant, and other transporters may participate in excretion, detoxification or self-defense. In *A. fumigatus*, GliA facilitates the excretion of gliotoxin. Similarly, the *tri12* gene contained in the trichothecene BGC encodes for a membrane protein required for the biosynthesis of trichothecene and virulence of *F. graminearum* on wheat crops (Chang et al., 2004; Menke

et al., 2012; Wang D.N. et al., 2014; Keller, 2015). Often, however, the deletion of the transporter gene in BGCs has no effect on production. Possibly, these metabolites are also recognized by other promiscuous transporters, or transporters that are not part of the BGC (Keller, 2015). For example, ZRA1 of *Gibberella zae,* whose gene is not localized to the zearalenona BGC, impacts zearalenone production. However, the expression of the *zra1* gene is regulated by the transcriptional factor ZEB2, whose gene localizes to the corresponding BGC (Lee et al., 2011). Also, the penicillin BGC of *P. chrysogenum* lacks a transporter gene whereas export of penicillin occurs against the concentration gradient, probably through the activity of multiple transporter proteins (van den Berg et al., 2008; Kistler and Broz, 2015). Furthermore, compartmentalization of the biosynthesis of penicillin is well documented requiring transport of penicillin precursors across the membrane of intracellular organelles (Weber et al., 2012a,b).

Other Genetic Engineering Strategies for the Activation of BGCs

Several approaches have been used to activate the expression of cryptic BGCs in a targeted manner. Usually, this is achieved by manipulation of pathway-specific regulatory genes, or by replacing endogenous promoters for inducible systems or strong promoters (Rutledge and Challis, 2015). The various approaches are summarized in **Figure 7**.

Manipulating biosynthetic pathways by genome editing

Due to the increasing number of sequenced filamentous fungi, it is necessary to make use of efficient genome editing tools to explore new potential sources of secondary metabolites. For many years, the unique strategy available for the genome edition of *P. chrysogenum* was based on the use of ku70/80 disrupted strains to improve the homologous recombination instead of the Non-Homologous End Joining (NHEJ) pathway (Weber et al., 2012a). This strategy allowed for the generation *P. chrysogenum* strains with high copy numbers of the penicillin cluster, the identification of a biosynthetic branch of the roquefortine cluster and the reactivation of the sorbicillinoid gene cluster (Nijland et al., 2010; García-Estrada et al., 2011; Ali et al., 2013; Ries et al., 2013; Salo et al., 2016; Guzmán-Chávez et al., 2017). Recently, a CRISPR/Cas9 based system was developed for genome modifications in *P. chrysogenum* (Pohl et al., 2016, 2018). This study demonstrated that the deletion of full gene clusters is feasible with minimal cloning efforts, which opens the possibilities to engineer new synthetic pathways and the re-factoring *P. chrysogenum* as platform organism.

Ribosome engineering

This approach has been applied for activating silent or poorly expressed BGCs (Ochi and Hosaka, 2013). Basically, this concept is derived from the activation of the actinorhodin BGC in *S. lividans* due to a point mutation in the *rpsL* gene, which encodes for the ribosomal S12 protein (Shima et al., 1996). Another successful examples in the BGCs activation have been reported by modifying the transcription and translation pathways via targeting different ribosomal proteins, RNA polymerases (RNAP) and translation factors (Ochi and Hosaka, 2013). In *P. purpurogenum* G59, a marine derived strain, the insertion of gentamicin resistance after treatment with high concentrations of this antibiotic, altered ribosomal functions of this fungus which allowed for the activation of dormant secondary metabolite gene clusters (Chai et al., 2012).

Heterologous expression and refactoring

Due to the broad range of molecular tools available to express heterologous pathways in yeast, several attempts have been undertaken to express NRPS and PKS genes with the remainder of the pathway in yeast (Rutledge and Challis, 2015). A recent study demonstrated that the baker's yeast *Saccharomyces cerevisiae* can be used as a platform to produce and secrete penicillin when the biosynthetic genes are expressed in this organism (Awan et al., 2016). Although the first step was performed when the acetyl-CoA:isopenicillin *N* acyltransferase (IAT), which catalyzes the last step in the penicillin biosynthesis was amplified from the *P. chrysogenum penDE* gene and expressed in *Hansenula polymorpha* (Lutz et al., 2005). However, most of the times the main obstacle is the large size (>40 kbp) of the DNA fragment that needs to be cloned, the effective activation/maturation of the expressed enzymes, and the toxicity of the produced compounds (Rutledge and Challis, 2015). Alternatively, fungi may be used as platform organism, as it was for instance demonstrated with the reconstruction of the citrinin gene cluster of *Monascus purpurea* in *A. oryzae* (Spraker and Keller, 2014). Likewise, the *in vivo* assembly of genetic elements has been successfully applied in *P. chrysogenum* through the overlapping of bi-partite fragments that reconstituted a functional *amdS* gene (marker), which eventually is integrated in the genome of this fungus proving the uncharacterized potential of *P. chrysogenum* as heterologous host (Polli et al., 2016). The potential of this approach follows a recent study employing *A. nidulans* as a host for the plasmid based expression of a diverse range of BGCs from other filamentous fungi (Clevenger et al., 2017).

The introduction of revolutionary new genetic tools, such as CRISPR/Cas9 offers more effective solutions to express specific BGCs. Such methods can contribute to product identification but also to the production of unique compounds by introduction of specific tailoring enzymes. These are the main strategies that are used for the activation of silent BGCs or for the modification/redirection of known biosynthetic pathways in order to increase NP diversification (Smanski et al., 2016). Specifically, this involves the expression of pathways from a plasmid in a suitable production host and a screen for product formation.

CONCLUDING REMARKS

For many years *P. chrysogenum* has been used as one of the main industrial strains to produce penicillins (β-lactams). Its genome sequence revealed an unexplored potential of *P. chrysogenum* as a source of NPs (van den Berg et al., 2008). Despite the development of bioinformatics tools for genome mining of BGCs to identify novel molecules (Blin et al., 2017), the experimental validation of product structure and identity is still

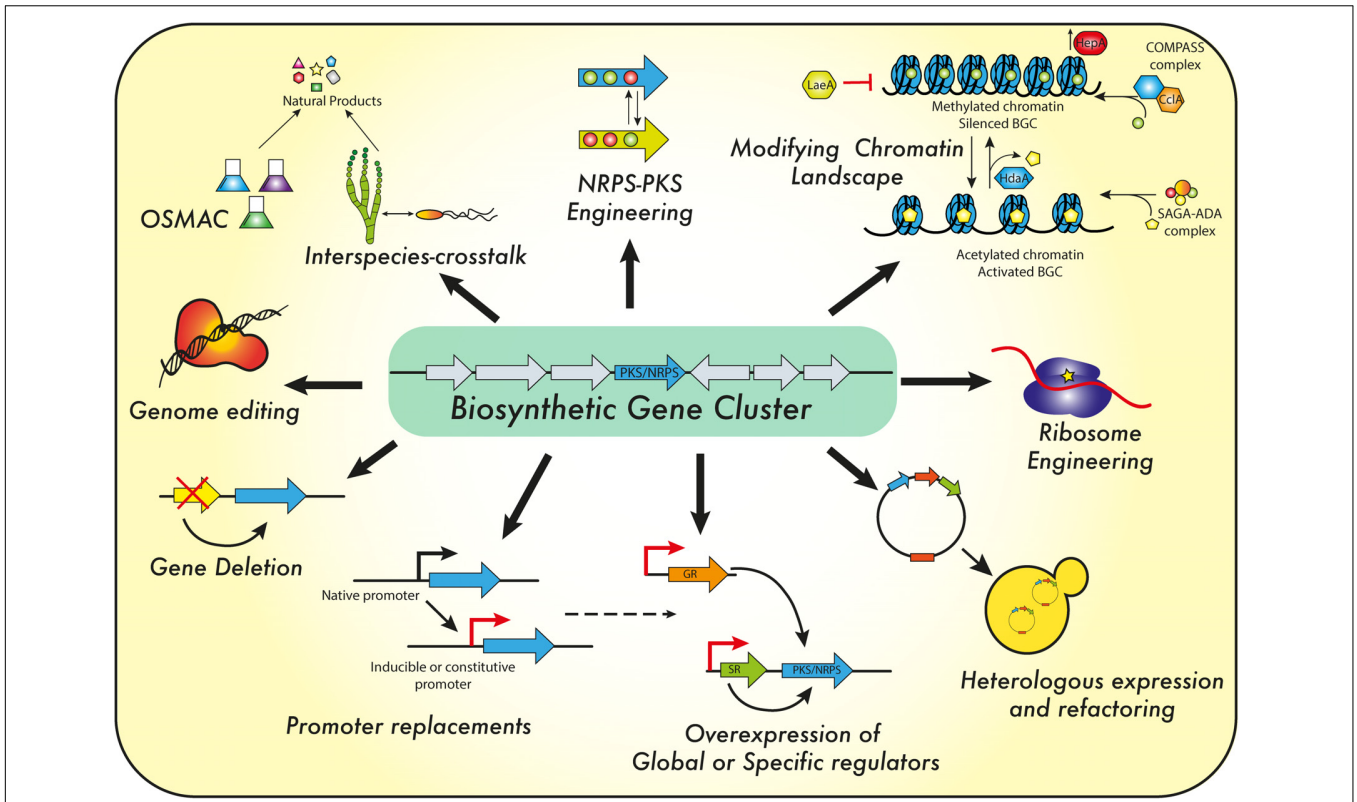

FIGURE 7 | Regulatory targets and strategies to engineer *P. chrysogenum* and other filamentous fungi for secondary metabolite formation. For details see the text.

necessary. However, most of the secondary metabolite associated genes in *P. chrysogenum* are silent or poorly expressed. Given the urgent need for new molecules based on novel chemical scaffolds for the use in the medical and biotechnological fields (e.g., antibiotics, anti-cancer agents, antivirals, nutraceuticals, pigments, surfactants and many more), the use of organisms that have been genetically domesticated offers a promising target solution for NP discovery due to the availability of molecular tools for their genetic modification. Here, we have summarized the main approaches that have been applied for *P. chrysogenum* and other filamentous fungi to bioengineer secondary metabolite BGC pathways which have led to a greater understanding of the main obstacles to be overcome to use this fungus as a generic cell factory. We discussed the main characteristics of the building enzymes (PKS and NRPS) in filamentous fungi. Despite the apparent modular organization, the complexity of these mega enzymes and the inherent interactions between the various domains within their structures has not allowed a straight forward approach for the PKS/NRPS engineering (Thirlway et al., 2012; Zhang et al., 2013; Weissman, 2016). However, the combinatorial swapping strategy of structural elements such as recognition regions has increased the perspectives for designing *de novo* biosynthetic pathways. Further research needs to be focused around PKS/NRPS engineering in filamentous fungi to facilitate the rational design of biosynthetic enzymes to produce another generation of novel compounds. To mine the secondary metabolome of filamentous fungi, general methods such as manipulating cultivation conditions have been used that can also be implemented as a high-throughput strategy. Another avenue is interfering with the genetic regulatory systems, either through the manipulation of specific or global regulators. This strategy also revealed crosstalk between certain BGCs and an important role of chromatin remodeling in BGC expression. Because of its pleiotropic effect that leads to the activation or silencing of biosynthetic pathways, chromatin remodeling might be used as a more general strategy to explore the production of new metabolites in filamentous fungi (Guzmán-Chávez et al., 2018).

A further major advance is the development of genome-editing tools that allow for efficient genetic engineering of complex fungal cell factories (Jakoèiunas et al., 2016). In *P. chrysogenum*, improved methods for homologous recombination and CRISPR/Cas9 as genome editing tool now facilitates more advanced engineering of this fungus (Pohl et al., 2016, 2018). This is further stimulated by the development of a synthetic biology toolbox using promoters and terminators as building blocks and more complex regulatory devices to control the expression of genes. Importantly, the *in vivo* assembly of genetic elements in *P. chrysogenum* offers a promising tool to build entire pathways from scratch with reducing cloning efforts at minimal costs (Polli et al., 2016). In particular the low cost of DNA synthesis will allow rapid progress using such approaches as exemplified by a study using *A. nidulans* as a host where a diverse set of BGCs was expressed from an

extra-chromosomal vector, the AMA plasmid (Clevenger et al., 2017). A further challenge is the generation of a platform strain in which endogenous BGCs have been removed to allow for more optimal carbon and nitrogen flow toward the production of the compounds of interest. Herein, the CRISPR/Cas9 based methods are instrumental (Pohl et al., 2016, 2018). Such a *Penicillium* platform might be used as a heterologous host to express a vast arsenal of BGCs from others filamentous fungi and represents a good alternative to yeast as expression host, an organism that does not naturally produce NRP and PK. The use of a such industrial strains to rapidly achieve the high level production of a novel metabolite has proven to be successful for pravastatin production (McLean et al., 2015) but a further step is to make use of secondary metabolite deficient industrial strains.

Despite the progress in genetic engineering and bioinformatics tools to identify BGCs, the main bottleneck to identify potentially interesting compounds has not yet been solved. Bioinformatic tools perform poorly in the prediction of the structures formed, and therefore future discovery programs will mostly dependent on high throughput methods to express foreign pathways and then use advanced metabolomics to identify the novel products. Since such approaches depend on high throughput, further efforts are needed to implement high throughput cloning methods to *P. chrysogenum* which will enable further studies to harness the enormous untapped source for NPs hidden in fungal (meta-)genomes.

AUTHOR CONTRIBUTIONS

FG-C and RZ wrote the manuscript. AD supervised, conceived, and designed the manuscript. RB co-supervised the manuscript.

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Engineered *Streptomyces lividans* Strains for Optimal Identification and Expression of Cryptic Biosynthetic Gene Clusters

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Streptomyces lividans is a suitable host for the heterologous expression of biosynthetic gene clusters (BGCs) from actinomycetes to discover "cryptic" secondary metabolites. To improve the heterologous expression of BGCs, herein we optimized *S. lividans* strain SBT5 via the stepwise integration of three global regulatory genes and two codon-optimized multi-drug efflux pump genes and deletion of a negative regulatory gene, yielding four engineered strains. All optimization steps were observed to promote the heterologous production of polyketides, non-ribosomal peptides, and hybrid antibiotics. The production increments of these optimization steps were additional, so that the antibiotic yields were several times or even dozens of times higher than the parent strain SBT5 when the final optimized strain, *S. lividans* LJ1018, was used as the heterologous expression host. The heterologous production of these antibiotics in *S. lividans* LJ1018 and GX28 was also much higher than in the strains from which the BGCs were isolated. *S. lividans* LJ1018 and GX28 markedly promoted the heterologous production of secondary metabolites, without requiring manipulation of gene expression components such as promoters on individual gene clusters. Therefore, these strains are well-suited as heterologous expression hosts for secondary metabolic BGCs. In addition, we successfully conducted high-throughput library expression and functional screening (LEXAS) of one bacterial artificial chromosome library and two cosmid libraries of three *Streptomyces* genomes using *S. lividans* GX28 as the library-expression host. The LEXAS experiments identified clones carrying intact BGCs sufficient for the heterologous production of piericidin A1, murayaquinone, actinomycin D, and dehydrorabelomycin. Notably, due to lower antibiotic production, the piericidin A1 BGC had been overlooked in a previous LEXAS screening using *S. lividans* SBT5 as the expression host. These results demonstrate the feasibility and superiority of *S. lividans* GX28 as a host for high-throughput screening of genomic libraries to mine cryptic BGCs and bioactive compounds.

Keywords: optimal hosts, global regulatory genes, heterologous expression, biosynthetic gene clusters (BGCs), secondary metabolites, library expression and function-directed screening system (LEXAS)

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INTRODUCTION

Microbial secondary metabolites display tremendous diversity in chemical structure and bioactivity and play an important role in drug discovery and development (Cragg and Newman, 2013). In recent years, the exploitation of the potential of "cryptic" biosynthesis, in the form of biosynthetic gene clusters (BGCs), in microbial genomes has become the focus of natural product research (Scherlach and Hertweck, 2009). Heterologous expression of BGCs is now an important technology for genome mining, biosynthetic study, and metabolic engineering. In addition, genome mining and biosynthetic studies on slow-growing or uncultured microorganisms can only be carried out through heterologous expression (Ongley et al., 2013). Many *Streptomyces* strains have the advantages of rapid growth and simple genetic manipulation. Moreover, as *Streptomyces* spp. and closely related actinomycetes are rich in secondary metabolite resources and therefore have the ability to provide precursors and cofactors required for efficient biosynthesis, engineered *Streptomyces* strains are highly suitable hosts for the heterologous expression of BGCs (Martinez et al., 2004; Wenzel and Müller, 2005). Excellent hosts must also be able to express all of the enzymes of the candidate biosynthetic pathway efficiently, including gene transcription, translation, and post-translational modifications, in order to successfully produce the corresponding compounds (Baltz, 2010). Ideally, endogenous secondary metabolic pathways should also be deleted to make a clean metabolic background and avoid substrate competition between endogenous and heterologous pathways (Komatsu et al., 2010). Such optimized hosts include *Streptomyces coelicolor* M1152, M1154, *Streptomyces avermitilis*, and *Streptomyces albus*, which has a naturally minimized genome (Gomez-Escribano and Bibb, 2011; Komatsu et al., 2013; Kallifidas et al., 2018).

Streptomyces lividans, a species closely related to *S. coelicolor*, has additional advantages as an expression host since it does not restrict (cleave) exogenous methylated DNA, whereas most actinomycetes such as *S. coelicolor* and *S. avermitilis* cleave methylated plasmid DNA from most *Escherichia coli* strains (MacNeil, 1988). Furthermore, when used as a recipient for *E. coli–Streptomyces* intergeneric conjugation, *S. lividans* exhibits a high efficiency of conjugative transfer, and this advantage is particularly important for experiments that require high-throughput transfer of arrayed library clones for screening genomic or metagenomic libraries by the function of unknown compounds (Wang et al., 2000). Indeed, an *S. lividans* TK24-derived strain was chosen as the expression host for the expression and screening of a metagenomic BAC library (Martinez et al., 2004). On the other hand, the wild-type strains of *S. lividans,* such as strain 1326, have disadvantages as they contain endogenous BGCs for secondary metabolites, such as *act* (for actinorhodin), *red* (for streptorubin or undecylprodigiosin) (Liu et al., 2017), and *cda* [for calcium-dependent antibiotic (CDA)]. Of more concern, such wild-type strains do not produce corresponding antibiotics under most culture conditions, implying that at least these BGCs are silent in the wild-type host (Hu et al., 2002).

Streptomyces lividans TK24 is a spontaneous *rpsL*[K88E] mutant that increases the production of actinorhodin; *rpsL*[K88E] has been shown to induce global upregulation of secondary metabolite biosynthesis (Shima et al., 1996; Okamoto-Hosoya, 2003). Additionally, the global regulatory genes $afsRS_{cla}$ from *S. clavuligerus* significantly promote the synthesis of actinorhodin, streptorubin, and CDA in *S. lividans* TK24 (Chen et al., 2012). On the basis of the above findings, we knocked out the *act*, *red*, and *cda* BGCs from *S. lividans* TK24 and inserted 1–2 copies of $afsRS_{cla}$, obtaining *S. lividans* SBT5 and SBT18 (Bai et al., 2014; Xu et al., 2016). Using these strains as expression hosts, Xu et al. (2016) optimized the previous functional genomic screening protocol (Martinez et al., 2004) and developed a library heterologous expression and function-directed screening system (LEXAS) for the screening of BGCs. LEXAS has facilitated the activation of cryptic BGCs and helped in mining compounds and new BGCs using the genomic libraries of *Streptomyces* spp. (Gao et al., 2017; Zheng et al., 2017; Chen et al., 2018).

In this study, in order to further improve the expression efficiency of heterologous BGCs and improve the screening efficiency of biologically active natural products by LEXAS technology, we optimized *S. lividans* SBT5 using a number of global positive and negative regulatory genes and genes encoding drug efflux pumps. We also demonstrated the superiority of the new strains in expression of heterologous BGCs and for LEXAS screening of cosmid and BAC libraries.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

Streptomyces lividans SBT5 [*S. lividans* TK24 Δ*act*Δ*redKL* Δ*cdaPS3-SLI3600*::$afsRS_{cla}$] (Bai et al., 2014) was used as the parent strain to construct optimized hosts for the heterologous expression of BGCs. *Streptomyces griseoruber* Sgr29 and *Streptomyces galtieri* Sag48 were isolated from Shennongjia (Eastern Hubei, China) forest soil (China Center for Type CultureCollection, CCTCC). *Streptomyces parvulus* 10 was isolated from the marine sponge *Phyllospongia foliascens* collected from Yongxing Island (South China Sea). *S. lividans* TK24 was used to construct the $wblA_{sl}$ knockout plasmid, and *S. coelicolor* M1154 (Gomez-Escribano and Bibb, 2011) was used as the cloning template for $nusG_{sc}$. Mannitol soy flour agar (MS) was used for sporulation of *Streptomyces* spp. Liquid culture was performed in TSBY medium containing 3% tryptone soy broth medium, 0.5% yeast extract, and 10.3% sucrose (Kieser et al., 2000). Agar media YBP (Ou et al., 2009), R3 (Shima et al., 1996), GYM (Ochi, 1987), No18 and No24 (Farnet et al., 2008) were used for the fermentation of *Streptomyces*. *Streptomyces* cultures were grown and fermented at 30°C. To determine growth curves for recombinant strains of *S. lividans*, strains were cultured in baffled flasks at 30°C with TSBY liquid medium (30 mL/flask).

Escherichia coli strains, *Staphylococcus aureus* CICC 10201, and *Bacillus mycoides* were cultured in Luria-Bertani (LB) medium at 37°C. *E. coli* XL1-Blue (Stratagene) was used as

the host for cosmid library construction, and *E. coli* DH10B (Invitrogen) was used for general cloning, plasmid maintenance, and as host for a BAC library. *E. coli* ET12567/pUB307 was used as a helper strain mediating tri-parental *E. coli–Streptomyces* intergeneric conjugation (MacNeil et al., 1992). *S. aureus* CICC 10201, *B. mycoides,* and *Saccharomyces sake* were used as indicator strains in the bioassay experiments.

pJTU2554 (Li et al., 2008) is a pSET152-derived, triplet COS site-bearing vector used to construct genomic cosmid libraries of *S. galtieri* Sag48 and *S. parvulus* 10. SuperCos 1 (Stratagene) was used to construct the genomic cosmid library of *S. lividans*. pHL921 (Xu et al., 2016) was the vector for the genomic BAC library of *S. griseoruber* Sgr29. pJTU2554-, pHL931-, and pHL921-derived clones carry the *attP* and *int* loci of the *Streptomyces* temperate phage ΦC31, and therefore can integrate into *Streptomyces* genomes at the *attB* site (Combes et al., 2002). pMS82, which bears the integration site *attP* and *int* loci of the *Streptomyces* temperate phage ΦBT1, was used as an integrative vector to carry genes of interest into the chromosome of *S. lividans* (Gregory et al., 2003). pUB307 is an RK2-derived, self-mobilizable plasmid that facilitates the intergeneric conjugation of $oriT_{RK2}$-plasmids from *E. coli* to *Streptomyces* (Bennett et al., 1977). pIJ773 and pIJ778 were used as templates for PCR amplification of *aac(3)IV* and *aadA* resistance markers, respectively (Gust et al., 2003). pHL851 (Chen et al., 2012) was the source of $afsRS_{cla}$.

Chemicals

The actinomycin D standard was purchased from Aladdin Bio-Chem Technology, Co. Standard compounds of murayaquinone, hybrubin A, dehydrorabelomycin, and piericidin A1 were prepared as described (Liu et al., 2012, 2018; Zhao et al., 2016; Gao et al., 2017).

Construction of Integrative Plasmids Carrying $nusG_{sc}$, Efflux Pump Genes, and $afsRS_{cla}$

A 1.4 kb fragment containing $nusG_{sc}$ was amplified by PCR using *S. coelicolor* M1154 genomic DNA as template and primers nusG-R (5′-CTAGTTCTTCTGGATCTGGTGCTTG-3′) and nusG-F (5′-GTGACGGACGCCGTGGGCTCCA-3′), and then cut with *Xba*I and ligated with pMS82 to yield pJTU6725. Two codon-optimized genes, $mdfA_{co}$ and $lmrA_{co}$, were synthesized based on the protein sequences of MdfA of *Enterobacteriaceae* (AFH35853) and LmrA of *Lactococcus lactis* subsp. *cremoris* MG1363 (CAL98427.1), respectively, with the codon usage table of *S. coelicolor*, and following the Codon Adaptation Tool[1]. The $mdfA_{co}$ gene has an overall GC content of 67% and a GC content of 99.5% for the third codon position. The $lmrA_{co}$ gene has an overall GC content of 65% and a GC content of 99.5% for the third codon position. The optimized $lmrA_{co}$-$mdfA_{co}$ DNA fragment was synthesized at HongXun Biotechnology, Co., Ltd., and ligated into pJTU6725 to construct pJTU6727. The paired genes $afsRS_{cla}$ (SCLAV_3382, SCLAV_3383) and the upstream *ermE** promoter in pHL851 were cloned into pJTU6727 to construct pJTU6728. The plasmids pJTU6725, pJTU6727, and pJTU6728 retain the hygromycin resistance gene (*hyg*), origin of transfer ($oriT_{RK2}$), and the *attP* and *int* loci of *Streptomyces* phage ΦBT1 from the integrative vector pMS82.

Construction of Genomic Cosmid Libraries of *Streptomyces* spp.

The genomic cosmid library of *S. lividans* TK24 was constructed using XL1-Blue as a host and SuperCos 1 as a vector according to the standard protocol (Kieser et al., 2000). The *S. lividans* TK24 genomic DNA was extracted and partially digested with *Sau*3AI. The 40–60 kb fragments were isolated by pulsed-field gel electrophoresis (PFGE), dephosphorylated, and ligated into the SuperCos 1 vector. The ligation product was packaged with λ phage packaging protein, transferred into *E. coli* XL1-Blue, and transformants were selected by kanamycin. The cosmid clones were extracted and digested with *Eco*RI and *Bam*HI to verify that the average inserted exogenous fragment size was 39 kb. The genomic cosmid libraries of *S. galtieri* Sag48 and *S. parvulus* 10 were constructed using *E. coli* XL1-blue MR/pUZ8002 as a host and pJTU2554 as a vector as described (Chen et al., 2012).

$wblA_{sl}$ Knockout of *S. lividans* SBT5

The genomic cosmid library of *S. lividans* TK24 was screened by PCR amplification using primers wblA-F (5′-CGTCC TCAACTGGCGGCGGTGAAT-3′) and wblA-R (5′-GGCCCC TGATCCGGCCTCGGGGCT-3′), and the cosmid clone 10H1 containing $wblA_{sl}$ was obtained. The $wblA_{sl}$ gene knockout plasmid was then constructed using 10H1 according to a PCR-targeting protocol (Gust et al., 2003). Firstly, the $wblA_{sl}$ in 10H1 was replaced by an *aac(3)IV* cassette amplified from pSET152 by λ-Red recombination. The resulting plasmid 10H1-Δ*wblA::aac(3)IV* was then transformed into *E. coli* DH5α containing the recombinant plasmid BT340, which expresses the FLP recombinase gene, to remove the *aac(3)IV* cassette by FLP recombination (Gust et al., 2003), yielding 10H1-Δ*wblA*. The *bla* (ampicillin resistance gene) on the backbone of 10H1-Δ*wblA* was then replaced by the *aac(3)IV-oriT* cassette amplified from pIJ773 by λ-Red recombination, resulting in the $wblA_{sl}$ knockout plasmid pHLJ42. The $wblA_{sl}$ gene in *S. lividans* SBT5 was deleted by homologous recombination between pHLJ42 and the chromosomal DNA. pHLJ42 contains 25.7 and 14.7 kb regions of *S. lividans* chromosomal DNA flanking either side of the mutated $wblA_{sl}$ locus. When pHLJ42, which does not contain an autonomous replication region or integration locus, was introduced into *S. lividans* SBT5 by conjugation, the apramycin-resistant (Apr^R) exconjugant should be a single-crossover mutant. To identify double-crossover mutants, the offspring colonies from the single-crossover mutant were screened for the loss of apramycin resistance, indicating the loss of *aac(3)IV*. A double-crossover mutant strain *S. lividans* SBT5Δ*wblA*, i.e., a $wblA_{sl}$ mutant, was confirmed by PCR (herein renamed *S. lividans* LJ101).

[1]http://www.jcat.de

Conjugation Using Mycelia as Recipient

Conjugation using mycelia was conducted following literature (Du et al., 2012). *S. lividans* LJ1018 was grown in 30 mL TSB liquid medium in a baffled flask, shaking at 180 rpm, 28°C for 48 h. The mycelia was collected by centrifugation at 5,000 rpm and washed with equal volume of 10% of glycerol once and 2× YT twice. Then 0.6 mL of washed mycelia was resuspended in 0.3 mL of 2× YT in an Eppendorf tube, mixed with 0.3 mL exponential phase donor *E. coli* cells. The mixture was spun at 5,000 rpm for 10 s, and the precipitate was spread on MS agar plate.

High-Throughput Screening (LEXAS) of *Streptomyces* Antibiotic BGCs Using *S. lividans* GX28 as the Library Expression Host

The high-throughput, tri-parental *E. coli–Streptomyces* conjugation of an arrayed genomic library, high-throughput fermentation, and bioactivity assay were carried out according to the LEXAS procedure (Xu et al., 2016). Cosmid or BAC libraries in *E. coli* DH10B in the format of 96-well plates were used as the arrayed donors for conjugation. Spores of *S. lividans* GX28 were used as recipients, and *E. coli* ET12567/pUB307 was used as the helper strain. The *E. coli* strains containing cosmid or BAC clones were cultured in LB liquid medium (150 μL/well), supplemented with apramycin, at 37°C overnight, and then transferred to antibiotic-free LB, cultured for 4–6 h until the optical density at 600 nm (OD_{600}) reached 0.4 to 0.6. *E. coli* ET12567/pUB307 (helper strain) was cultured in LB (120 mL/library) containing a final concentration of 50 μg/mL chloramphenicol at 37°C until the OD_{600} was between 0.4 and 0.6. The cells were then collected by centrifugation and resuspended in 20 mL LB medium. Next, 20 μL of ET12567/pUB307 was pipetted into each well of the 96-well plates in which the BAC/cosmid library was inoculated, and the plates were shaken on a rotary shaker at 200 rpm for 5 min to allow thorough mixing. *S. lividans* GX28 (recipient) was grown on MS sporulation medium for 5–6 days at 30°C. The fresh spores were collected and resuspended in 4 mL of 2× YT medium, heat-shocked at 50°C for 10 min, and then spread on MS plates supplemented with Mg^{2+} (20 mM). The donor-helper *E. coli* mixtures were replicated from 96-well plates onto spore-coated MS plates using a 48-pin replicator. After incubation at 30°C for 12 to 16 h, the MS plates were covered with apramycin and trimethoprim to final concentrations of 50 μg/mL to inhibit the *E. coli* strains. The exconjugants were cultured for another 4–6 days and then replicated to MS plates containing final concentrations of 50 μg/mL apramycin and 25 μg/mL nalidixic acid to remove the residual *E. coli*. The *S. lividans* GX28 exconjugants were fermented and subjected to high-throughput screening based on antibacterial activity. The *S. lividans* GX28 exconjugants of libraries were replicated to the agar fermentation media YBP, R3, GYM, No18, and No24 by replicator and cultured at 30°C for 7 days. The surface of the fermentation media were covered with soft agar premixed with indicator bacteria, and the inhibition zones produced by heterologous expression of the active compounds were observed after 1–2 days of incubation.

Sequence Analysis

The sequences of both ends of the inserts in BAC clones were determined with primers pHL921F (5′-ATGTTTTT CGTCTCAGCC-3′) and pHL921R (5′-CCTTTAGTTG TTCCTTTC-3′). The end sequences of cosmid clones were determined with primers pJTU2554F (TGTAA AACGACGGCCAGT) and pJTU2554F (GGCACCTG TCCTACGAGTTG). The DNA end sequences were then mapped to the genomic sequences. The DNA sequences of BAC or cosmid inserts were submitted to antiSMASH[2] for the analysis of secondary metabolic BGCs.

Isolation and Analysis of Compounds

Actinorhodin was isolated and measured using a published method (Bystrykh et al., 1996). The *S. lividans* strains were cultured on solid YBP medium for 84 h, 500 mg agar culture was taken from each plates, 500 μL of 1 M NaOH was added, followed by crushing using a homogenizer (5,000 rpm, 15 s; twice). The samples were centrifuged at 12,000 × *g*, 5 min and the absorbance of the supernatants was measured at 633 nm. The isolation and analysis of piericidin A1, murayaquinone, dehydrorabelomycin, and actinomycin D, followed a similar approach as the following. Fermented culture (40 mL) was extracted three times with ethylacetate (150 mL). The combined extracts were concentrated on a rotary evaporator (Buchi R210) at 37°C and then dissolved in 1 mL methanol. The crude extract (20 μL) was filtered and injected onto a C18 reversed-phase column (Agilent Zorbax ODS C18, 5 μm, 4.6 by 250 mm) and analyzed by high performance liquid chromatography (HPLC) in the Agilent 1260 HPLC system using mobile phase A (H_2O supplemented with 0.1% formic acid) and mobile phase B (acetonitrile) at a flow rate of 0.6 mL/min. The elution procedure was: 0–2 min, 5% B (and 95%A); 2–25 min, 5–40% B; 25–35 min, 40–100% B; 35–40 min, 100% B; 40–45 min, 100–5% B; 45–55 min, 5% B.

The isolation and identification of hybrubin A was carried out as described (Zhao et al., 2016). Hybrubin A was eluted using the following HPLC conditions: mobile phase A was H_2O (supplemented with 0.1% formic acid), mobile phase B was methanol; flow rate of 0.6 mL/min; 0 min, 40% B; 5–15 min, 65–80% B; 15–20 min, 80–100% B; 20–25 min, 100% B; 25–26 min, 100–40% B; 26–35 min, 40% B.

Agilent G6530 HR ESI-QTOF mass spectrometry equipped with Agilent 1260 HPLC system was used to identify piericidin A1, dehydrorabelomycin, murayaquinone, actinomycin D, and hybrubins.

RESULTS

Engineering of *S. lividans* SBT5-Derived Strains Using Global Regulatory Genes

The $nusG_{sc}$ gene of *S. coelicolor* A3(2) encodes an anti-terminator that is functionally conserved in prokaryotes, eukaryotes, and archaea (Mason and Greenblatt, 1991; Burmann et al., 2010).

[2]http://antismash.secondarymetabolites.org/

For cloning this gene with its native promoter, we amplified a 1.4 kb fragment containing the $nusG_{SC}$ coding region and the 536 bp upstream region by PCR, and then the fragment was ligated into pMS82 to yield the integrative plasmid pJTU6725 (**Figure 1A**). pJTU6725 was conjugated to *S. lividans* SBT5 to generate *S. lividans* GX25, in which the plasmid is integrated into the genome at the $attB^{\Phi BT1}$ site.

The *lmrA* gene in *Lactococcus lactis* subsp. *cremoris* MG1363 encodes a multidrug resistance ABC transporter ATP-binding and permease protein (van Veen et al., 1996), and the *mdfA* gene in *Escherichia coli* K-12 encodes a multidrug efflux transporter protein. Both *lmrA* and *mdfA* confer hosts with resistance to a variety of antibiotics by heterologous expression (Edgar and Bibi, 1997). The G+C contents of the original *lmrA* and *mdfA* genes were 39.0 and 52.4%, respectively. For the expression of these two multidrug resistance genes in the high G+C content genome of *S. lividans*, we synthesized the codon-optimized twin gene cassette $lmrA_{co}$-$mdfA_{co}$ based on the protein sequences and the degenerate codon usage table of the *S. coelicolor* genome. The promoter of the non-ribosomal peptide synthase (NRPS) gene *cdaPS1* from the CDA BGC was placed upstream of $lmrA_{co}$ to control the expression of $lmrA_{co}$ and $mdfA_{co}$. The previously reported production of CDA in $afsRS_{cla}$-carrying *S. lividans* strains suggested that the P_{cdaPS1} promoter has been activated (Chen et al., 2012; Bai et al., 2014). The synthetic operon P_{cdaPS1}-$lmrA_{co}$-$mdfA_{co}$ was ligated to pJTU6725 to construct pJTU6727 (**Figure 1A**). The integrative plasmid pJTU6727 was conjugated to *S. lividans* SBT5 to yield *S. lividans* GX27.

The global transcriptional regulator $AfsR/S_{cla}$ from *S. clavuligerus* ATCC 27064 (NRRL3585) increased the production of actinorhodin and CDA in *S. lividans* TK24 (Chen et al., 2012). We cloned $afsR/S_{cla}$ and the $ermE^*$ promoter from pHL851 into pJTU6727 to construct pJTU6728 (**Figure 1A**), which was conjugated to *S. lividans* SBT5 to construct *S. lividans* GX28 (**Figure 1B**).

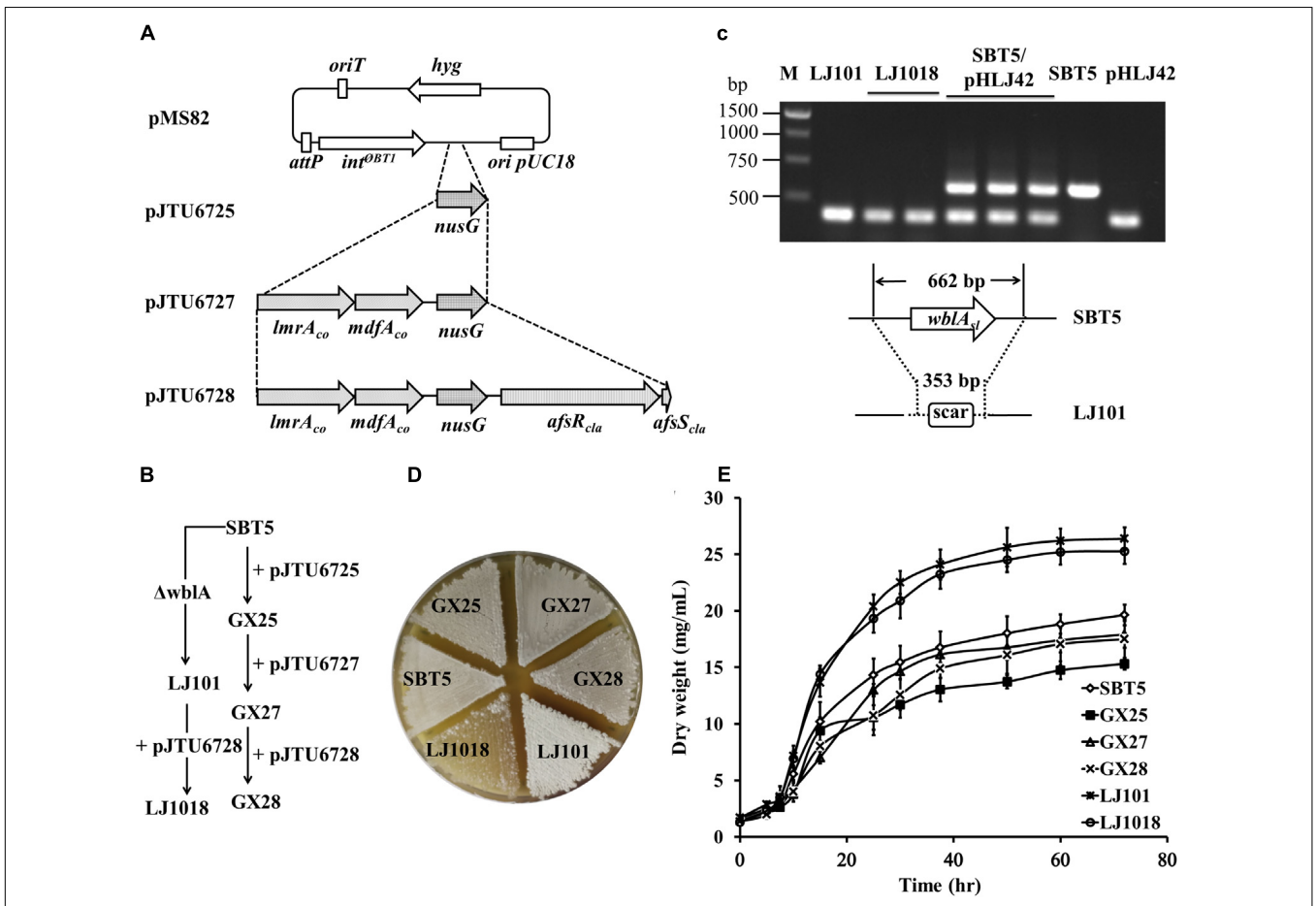

FIGURE 1 | Engineering of *Streptomyces lividans* strains. **(A)** Integrative plasmids derived from pMS82 carrying regulatory genes and codon-optimized multidrug resistance transporter genes. **(B)** Construction of the engineered *S. lividans* strains from SBT5. **(C)** PCR confirmation of the $wblA_{sl}$ deletion mutants LJ101 and LJ1018. M, 1 kb ladder. pHLJ42, cosmid containing a deleted $wblA_{sl}$ locus; SBT5/pHLJ42, single-crossover mutant; SBT5, the parent strain. **(D)** Growth of the engineered strains and SBT5 on MS medium at 30°C for 72 h. LJ101 is white, and LJ1018 is bald. **(E)** Biomass accumulation of the engineered *S. lividans* strains and SBT5 over 72 h of cultivation in TSBY liquid medium. Spores (or mycelium of LJ101 and LJ1018) were pre-cultured on TSBY at 30°C for 48 h. An aliquot of the resultant vegetative culture was diluted to 100-fold by 30 mL TSBY and shaken at 180 rpm, 30°C. A 1 mL culture sample was taken and centrifuged for 10 min at 12,000 rpm. Supernatants were discarded, and the pellet was dried at 80°C for 48 h and weighed.

The gene $wblA_{sl}$ (SLIV_20395) of *S. lividans* TK24 encodes a global transcriptional regulator of the WhiB family (Yu et al., 2014) and has 99% similarity to *S. coelicolor* $wblA_{sc}$ (SCO3579). To knock out $wblA_{sl}$ in *S. lividans* SBT5, the cosmid clone 10H1 containing $wblA_{sl}$ was obtained from a genomic cosmid library of *S. lividans* TK24. An in-frame deletion was made in $wblA_{sl}$ on 10H1 to construct the gene knockout vector pHLJ42, which contains $wblA_{sl}$ flanking sequences of 25.7 and 14.7 kb for homologous recombination. The $wblA_{sl}$-knockout strain LJ101 was constructed using pHL42 via homologous recombination and confirmed by PCR (**Figure 1C**). Compared with the parental strain SBT5, *S. lividans* LJ101 exhibited a "white" phenotype (**Figure 1D**): no spore pigment was produced and the white aerial hyphae did not develop into spores. This indicated that $wblA_{sl}$ plays an important role in aerial hyphae development similar to the *wblA* from *S. coelicolor* A3(2) (Fowler-Goldsworthy et al., 2011). pJTU6728 was conjugated to *S. lividans* LJ101 to yield *S. lividans* LJ1018, which displayed a "bald" phenotype with only sparse white mycelium (**Figure 1D**).

Growth curves indicated that the introduction of pJTU6725, pJTU6727, and pJTU6728 into *S. lividans* SBT5 did not significantly affect the growth and biomass accumulation of the host strain. However, the biomass of the $wblA_{sl}$ deletion strains *S. lividans* LJ101 and *S. lividans* LJ1018 was significantly improved. The dry weight of the two strains was 1.6 times higher than that of *S. lividans* GX28 after 72 h of culture (**Figure 1E**; $p < 0.0001$).

Heterologous Expression of the Pigmented Polyketide Antibiotic Actinorhodin in Engineered *S. lividans* Strains

To test the ability of the engineered *S. lividans* strains to express polyketide BGCs, we expressed the actinorhodin BGC using *S. lividans* GX25, *S. lividans* GX27, *S. lividans* GX28, *S. lividans* LJ1018, and the parent strain *S. lividans* SBT5 as the expression hosts. The *act* BGC is a 22 kb type II polyketide synthase (PKS) BGC, and actinorhodin (**Figure 2A**) is a pH-sensitive, pigmented aromatic polyketide antibiotic that is red at acidic pH and blue at alkali pH. Plasmid pMM1 (45 kb) carrying the complete *act* BGC (Zhou et al., 2012) was introduced into the *S. lividans* series of hosts by conjugation. High conjugation frequencies, ca. 10^{-2}/cfu, were observed when *S. lividans* GX25, *S. lividans* GX27, *S. lividans* GX28, and *S. lividans* SBT5 were used. Because *S. lividans* LJ1018 is deficient in sporulation, mycelium was used as the recipient for conjugation, and 100s of exconjugants were obtained on each conjugation plate, with a conjugation frequency of around 10^{-6}/cfu. After fermentation in YBP medium for 72 h, the blue color of actinorhodin was observed due to the heterologous expression of the *act* BGC in the exconjugants. Observation of the color of the YBP fermentation medium revealed that the heterologous expression of actinorhodin in the optimized hosts *S. lividans* GX25, GX27, GX28, and LJ1018 progressively increased compared to levels in SBT5 (**Figure 2B**). The yield of actinorhodin of GX25/pMM1 was 1.3 times higher than that of SBT5/pMM1 ($p < 0.001$), and the yields of actinorhodin in *S. lividans* GX27/pMM1, GX28/pMM1, and LJ1018/pMM1 were 12.8, 21.6, and 23.3 times higher than that of SBT5/pMM1, respectively ($p < 0.0001$; **Figure 2C**), indicating that the addition of $nusG_{sc}$, the drug efflux pump genes, and $afsRS_{cla}$ and the knockout of $wblA_{sl}$ in SBT5 up-regulated the production of actinorhodin.

Heterologous Expression of the Aromatic Polyketide Antibiotic Murayaquinone in the Engineered *S. lividans* Strains

Murayaquinone is a tricyclic, angular aromatic polyketide 9,10-phenanthraquinone antibiotic produced by a type II PKS

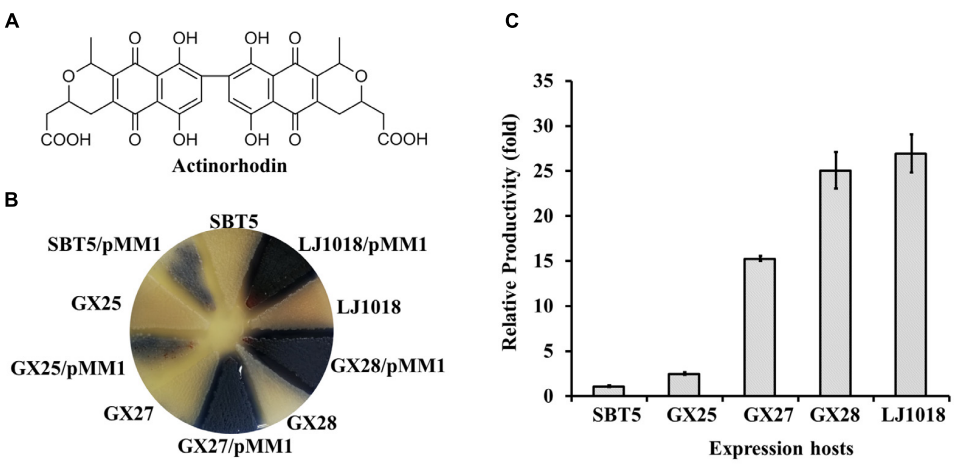

FIGURE 2 | Heterologous expression of actinorhodin by the engineered *S. lividans* strains carrying the *S. coelicolor* actinorhodin BGC on pMM1. **(A)** Structure of actinorhodin. **(B)** Heterologous expression of the actinorhodin BGC in *S. lividans* strains on YBP agar medium. The top of the culture plate after 72 h fermentation is shown. pMM1, plasmid carrying the *S. coelicolor* actinorhodin BGC. **(C)** Quantification of actinorhodin production by various expression hosts carrying pMM1 on YBP medium. The productivity related to *S. lividans* SBT5/pMM1 was present. Data are from three biological replicates.

pathway (**Figure 3A**, Gao et al., 2017). The murayaquinone BGC is about 56 kb and was cloned into BAC clone 3B4. 3B4 was obtained by screening the genomic BAC library of *S. griseoruber* Sgr29 using *S. lividans* SBT5 as the high-throughput heterologous expression host, conferring the exconjugants with antibacterial activity against *S. aureus* (Gao et al., 2017). The exconjugants carrying 3B4 were fermented on solidified media No18 and No24, and the crude extracts were analyzed by HPLC. Three new peaks were observed on the HPLC trace of *S. lividans* GX28/3B4 fermented on No18 medium, with the same retention time as the standard samples of murayaquinone and murayalactone 1, 2 (**Figure 3B**). Murayalactone 1 and 2 were the main products on No18 medium, while murayaquinone was the main product on No24 medium. The identity of murayaquinone and murayalactones isolated from *S. lividans* GX28/3B4 was further confirmed by high-resolution mass spectrometry (HR-MS) (**Figure 3C**). However, murayaquinone and murayalactones were not detectable by HPLC from the *S. griseoruber* Sgr29 fermentation culture (**Figure 3B**), which is consistent with the literature. To compare the production of murayaquinone, all exconjugants were fermented on No24, and the areas of murayaquinone peaks in the HPLC traces were measured. The yield of murayaquinone was extremely low (about 0.11 mg/L) in the parent host SBT5/3B4 but was significantly increased in all engineered hosts ($p < 0.05$). The yields of murayaquinone in *S. lividans* GX28/3B4 and *S. lividans* LJ1018/3B4 were much higher than that of the original host SBT5/3B4 (74 and 96 times higher, respectively, $p < 0.0001$), and the yield of murayaquinone in *S. lividans* LJ1018/3B4 was 10.6 mg/L (**Figure 3D**).

Heterologous Expression of Hybrubins in the Engineered *S. lividans* Strains

The *hbn* BGC from *Streptomyces variabilis* Snt24 is a small PKS-NRPS hybrid BGC responsible for the biosynthesis of 5-ethylidenetetramic acid (ETA); the truncated *red* pathway in *S. lividans* SBT5 synthesizes 4-methoxy-2,2′-bipyrrole-5-carbaldehyde (MBC), and condensation of ETA with MBC produces the "non-natural" red compounds named hybrubins (Zhao et al., 2016). pZZL3 is an integrative plasmid containing a 13 kb *hbn* BGC cloned from the *S. variabilis* Snt24 genome. The heterologous expression of *hbn* BGC in *S. lividans* SBT5 led to the production of the red-pigmented secondary metabolites hybrubin A-C (**Figure 4A**, Zhao et al., 2016). When the pZZL3-carrying exconjugants of *S. lividans* SBT5, GX25, GX27, GX28, and LJ1018 were fermented with R3 medium, red pigment was observed in the crude extract whereas the vector control did not produce red pigment (**Figure 4B**). HPLC analysis indicated that hybrubins A-C were produced and that hybrubin A was the main component (**Figure 4C**). The identity of hybrubin A was confirmed by HR-ESI-MS (**Figure 4D**). The relative yield of hybrubin A was evaluated based on the HPLC peak area.

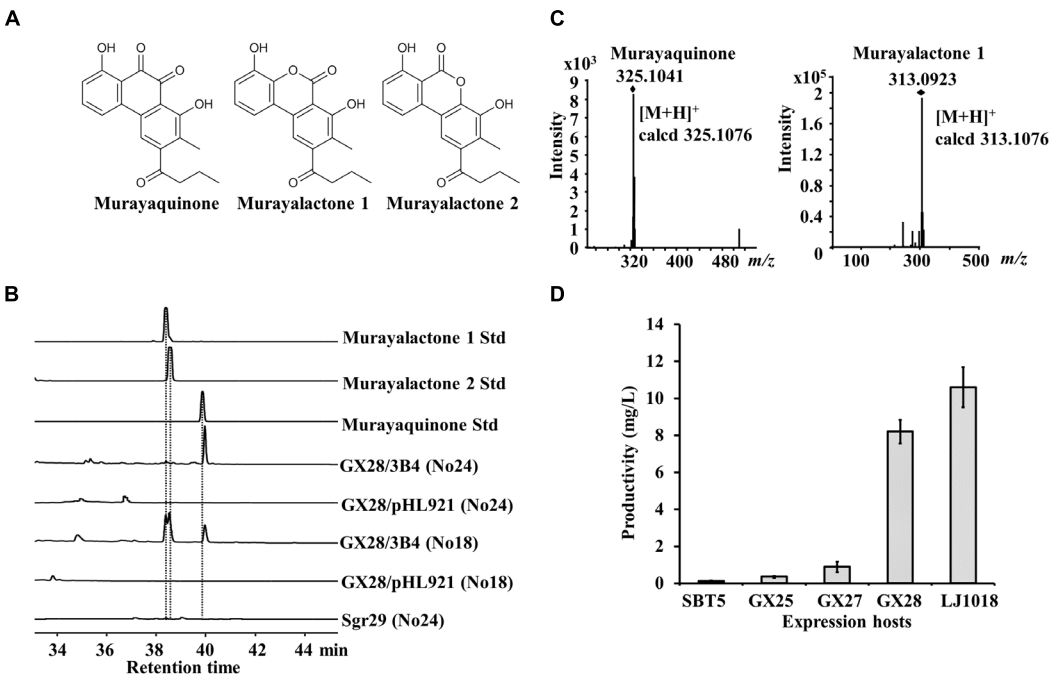

FIGURE 3 | Heterologous expression of the murayaquinone BGC in engineered *S. lividans* strains. **(A)** Structure of murayaquinone and murayalactones. **(B)** HPLC analysis of the exconjugants carrying the murayaquinone BGC from BAC 3B4 and of the original strain *Streptomyces griseoruber* Sgr29. The absorbance was measured at 350 nm. No18 and No24 agar media were used for fermentation. 3B4, a BAC clone containing the murayaquinone BGC. No murayaquinone or murayalactones were detected from *S. griseoruber* Sgr29. **(C)** HR-MS spectrum of murayaquinone and murayalactone 1 isolated *from S. lividans* GX28/3B4. The spectra of murayalactone 1 and 2 are identical. **(D)** Quantification of murayaquinone production on No24 medium by engineered *S. lividans* strains carrying 3B4. Data are from three biological replicates.

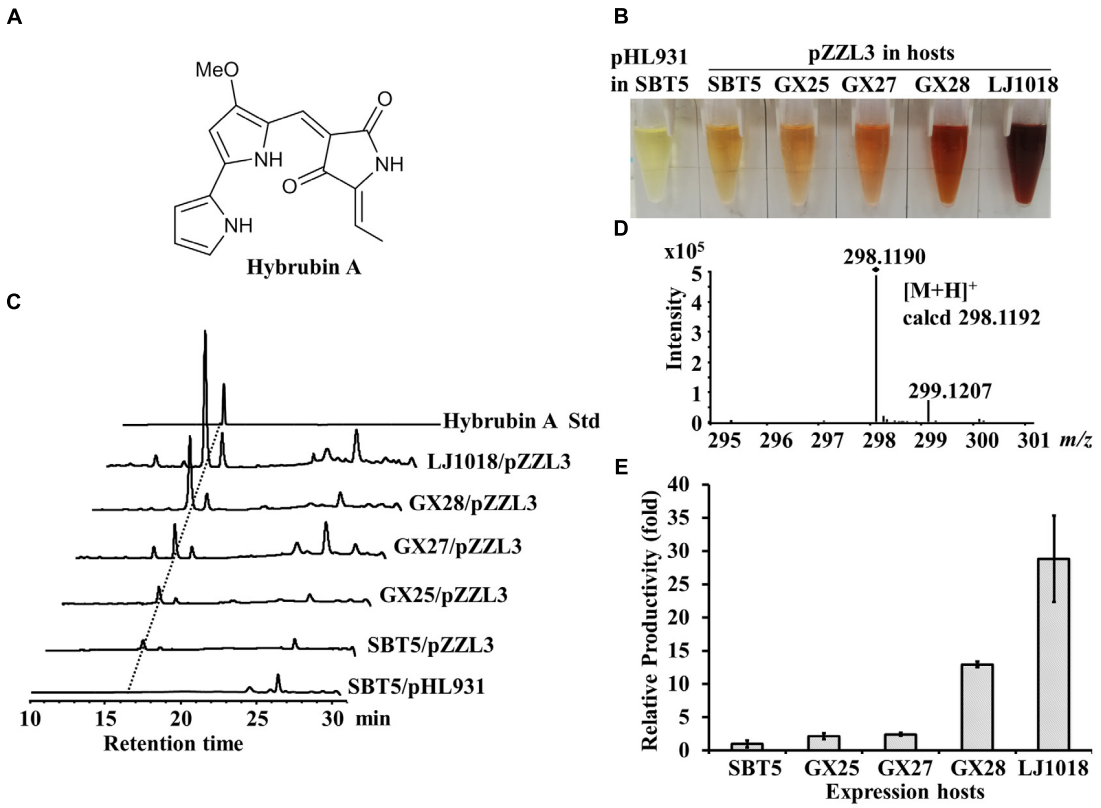

FIGURE 4 | Heterologous expression of hybrubin A by engineered *S. lividans* strains carrying pZZL3. **(A)** Structure of hybrubin A. **(B)** Ethyl acetate crude extracts of the exconjugants carrying pZZL3 fermented on R3 medium. pZZL3, a plasmid containing the tetramic acid (ETA) BGC; pHL931, the empty vector control. Red pigmented hybrubin A was observed in extracts of the pZZL3-carrying exconjugants. The vector control did not produce red pigment. **(C)** HPLC analysis of hybrubin A production by *S. lividans* strains. **(D)** HR-MS spectrum of hybrubin A isolated *from S. lividans* GX28/pZZL3. **(E)** Quantification of the heterologous expression of hybrubin A in R3 liquid medium. Yields of hybrubin A from the optimized hosts *S. lividans* GX28 and LJ1018 were much higher than from the original host *S. lividans* SBT5. Data are from three biological replicates.

The yield of hybrubin A in GX25/pZZL3 and GX27/pZZL3 was slightly higher than in SBT5/pZZL3 (2.2 and 2.5 times, respectively, $p < 0.05$), whereas the yield in GX28/pZZL3 and LJ1018/pZZL3 was greatly increased, reaching 13 times and 29 times the yield in SBT5/pZZL3, respectively (**Figure 4E**).

Discovery of a Piericidin A1 BGC Using LEXAS and *S. lividans* GX28

We tested the ability of the engineered strain *S. lividans* GX28 to serve as a host for LEXAS screening of antibiotics and their corresponding BGCs, using the *S. griseoruber* Sgr29 genomic BAC library, which contains 912 arrayed clones with an average insertion size of about 100 kb (Gao et al., 2017). *S. lividans* SBT5 had been used as a host for the high-throughput heterologous expression in a previous screening, and seven positive BAC clones with *S. aureus* resistance were obtained from this genomic BAC library, three of which contained the murayaquinone BGC (Gao et al., 2017). Using *S. lividans* GX28 as the expression host, nine new *S. aureus*-resistant positive BAC clones were obtained, five of which (4E6, 4F9, 1H5, 2D3, and 4G10) shared overlapping DNA regions (**Figure 5A**). The termini of these five BACs were sequenced with primers pHL921-F/R, and then the sequences were aligned with the *S. griseoruber* Sgr29 genomic sequence. The five BAC plasmids were found to have a 98 kb overlapping region, and analysis of this region by AntiSMASH revealed that it contains a 50 kb piericidin A1 BGC, which included six type I polyketide synthase (PKSI) genes and five post-modification genes highly homologous to piericidin A1 BGC genes in *S. piomogeues*. Piericidin A1 is an α-pyridone antibiotic (**Figure 5B**) that inhibits the mitochondrial respiratory chain and NADH-ubiquinone oxidase and exhibits weak antimicrobial and antitumor activities (Liu et al., 2012; Chen et al., 2014). To verify the function of the piericidin A1 BGC, one of the BAC clones, 1H5, was transferred to the expression host *S. lividans* GX28, and the exconjugants was fermented with R3 medium. The fermented culture of GX28/1H5 had inhibitory activity against *B. mycoides*, whereas the empty vector control (*S. lividans* GX28/pHL921) did not produce an inhibition zone (**Figure 5C**). HPLC and HR-MS analysis indicated that piericidin A1 was produced by GX28/1H5 and *S. griseoruber* Sgr29 (**Figures 5D,E**).

To detect the yield of piericidin A1 in different expression hosts, 1H5 was transferred into the five *S. lividans* hosts for heterologous expression, and the resulting exconjugants and

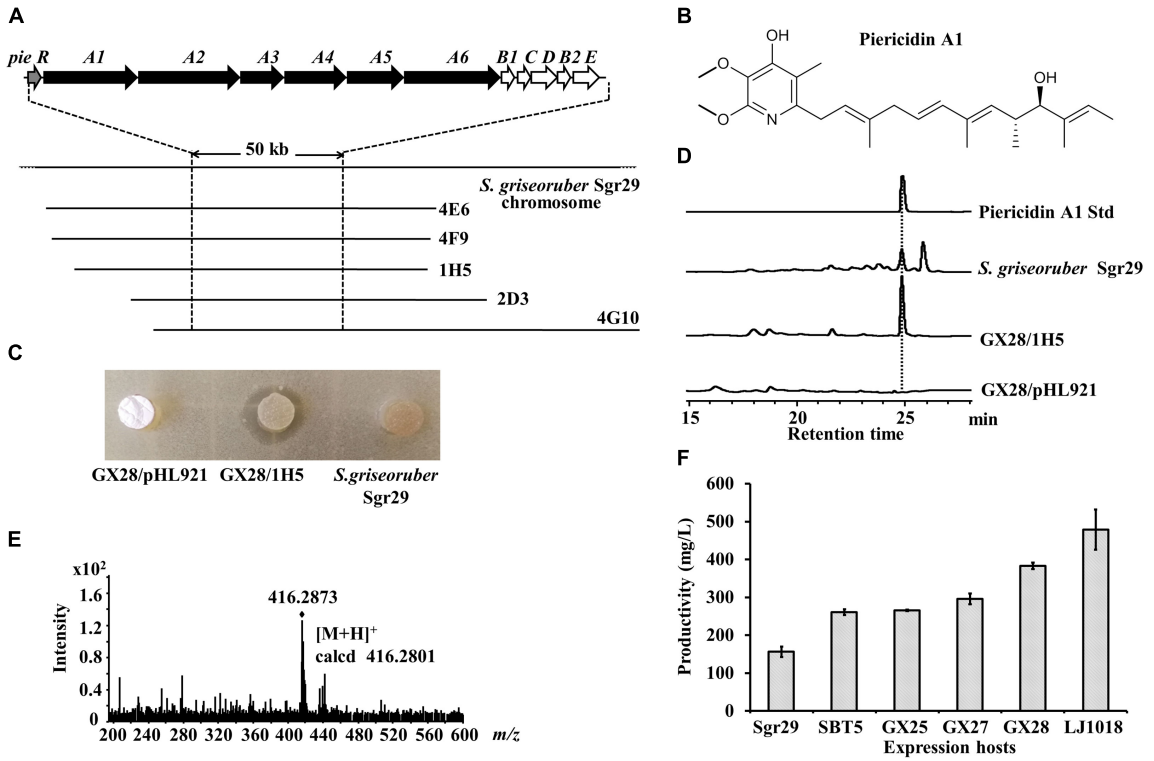

FIGURE 5 | Identification of piericidin A1 and the *pie* BGC by LEXAS screening of the *S. griseoruber* Sgr29 BAC genomic library using *S. lividans* GX28 as host. **(A)** Overlapping map of the five BAC clones containing the 50 kb piericidin A1 BGC. Thick arrows on the top line denote genes of the piericidin biosynthetic pathway. **(B)** Structure of piericidin A1. **(C)** Bioassay of the exconjugant *S. lividans* GX28/1H5 against *Bacillus mycoides*. Plugs of fermented culture were placed on the surface of agar medium inoculated with *B. mycoides*. The bioassay plate was incubated for 24 h at 37°C. A zone of inhibition was observed around the plug of GX28/1H5. No antibacterial activity was observed from *S. griseoruber* Sgr29 or the vector control. **(D)** HPLC analysis of the ethyl acetate extracts of fermented cultures of *S. lividans* GX28/1H5, the vector control, and *S. griseoruber* Sgr29. The absorbance was measured at 254 nm. **(E)** HR-MS spectrum of piericidin A1 isolated from *S. lividans* GX28/1H5. **(F)** Quantification of the production of piericidin A1 by Sgr29 and the 1H5-carrying exconjugants in five expression hosts on R3 agar medium.

the natural strain *S. griseoruber* Sgr29 were fermented. HPLC analysis showed that, although *S. griseoruber* Sgr29 produced high levels of piericidin A1 (156.6 mg/L), the yields resulting from heterologous expression in the *S. lividans* hosts were significantly higher ($p < 0.001$). The yield of piericidin A1 from *S. lividans* GX28/1H5 was 2.4 times that of *S. griseoruber* Sgr29, and the yield from *S. lividans* LJ1018/1H5 was even higher, at 3.1 times the yield from *S. griseoruber* Sgr29 and reaching 478 mg/L (**Figure 5F**).

Discovery of a Dehydrorabelomycin BGC Using LEXAS and *S. lividans* GX28

We constructed a genomic cosmid library of *S. galtieri* Sag48, a species isolated from forest soil by CCTCC, and performed LEXAS screening using *S. lividans* GX28 as the high-throughput heterologous expression host. The LEXAS screening identified an exconjugant displaying weak inhibition activity against *B. mycoides* and which contained cosmid plasmid 8F5. Sequencing analysis revealed that 8F5 has a 32 kb insertion sequence containing 27 genes having high level of similarity (81–95%) to the *alpA-alpW* genes in the type II polyketide BGC of kinamycin from *S. ambofaciens* (**Figure 6A**). The complete kinamycin BGC is 63 kb and cannot be packaged into a single cosmid clone (Wang et al., 2015; Liu et al., 2018). Although cosmid 8F5 contains PKS genes (*alpABC*) and early modification genes for the synthesis of kinamycin intermediates, it does not contain other genes required for the synthesis of the final product (i.e., kinamycin). To analyze the metabolites produced via this cosmid, 8F5 and the vector pJTU2554 were introduced into the five *S. lividans* hosts by conjugation, and the resulting exconjugants and the natural strain *S. galtieri* Sag48 were fermented on No18 agar plates. Extracts of the fermented cultures were analyzed by HPLC. The crude extract of *S. lividans* LJ1018/8F5 produced an absorption peak at 39 min, which was not produced by *S. galtieri* Sag48 and the vector control strain *S. lividans* LJ1018/pJTU2554 (**Figure 6B**). The compound was detected by LC-MS, and its molecular weight, with an *m/z* value of 321.0710, was consistent with that of dehydrorabelomycin (*m/z* of $[M+H]^+$ calcd. 321.0763) (**Figures 6C,D**), which is an intermediate of the kinamycin biosynthetic pathway.

Quantitative comparison of dehydrorabelomycin production indicated that *S. lividans* GX28/8F5 and LJ1018/8F5 yielded levels 6.7 times and 12.7 times, respectively, the amount

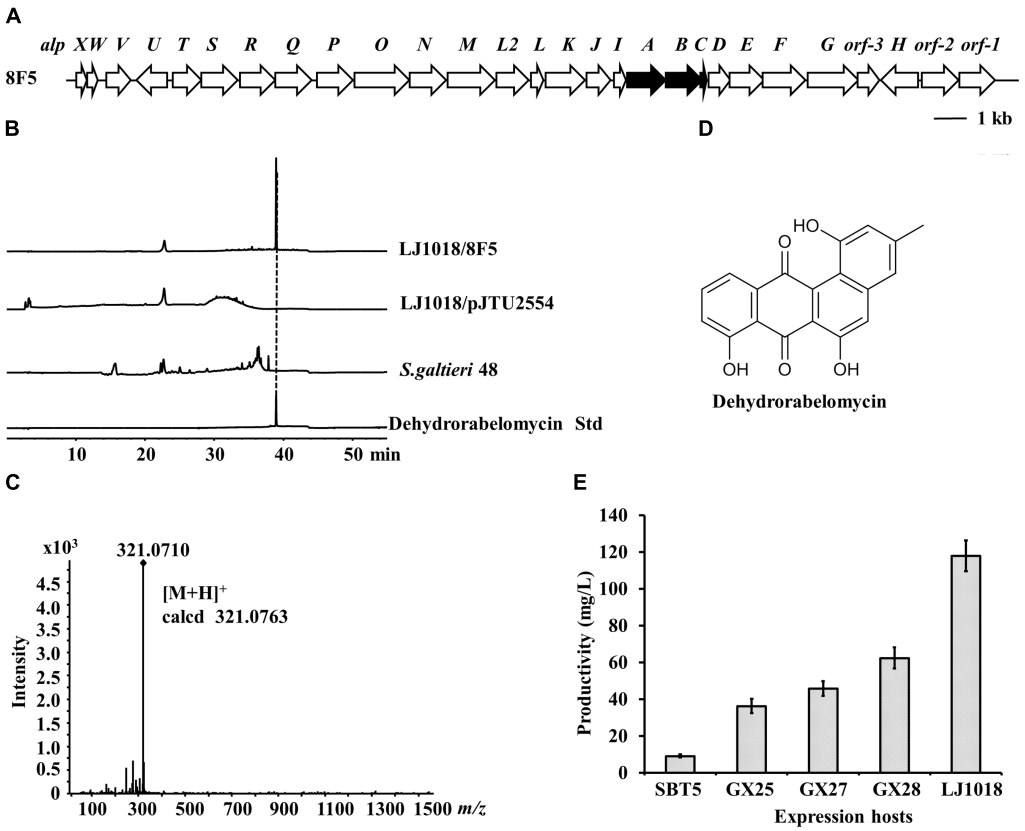

FIGURE 6 | Identification of dehydrorabelomycin and its BGC by LEXAS screening of the *Streptomyces galtieri* Sag48 genomic cosmid library using *S. lividans* GX28 as host. **(A)** Gene organization of the dehydrorabelomycin BGC in cosmid 8F5. The minimal *pks* genes are black. **(B)** HPLC analysis of ethylacetate extracts of fermented cultures. The absorbance was measured at 350 nm. **(C)** HR-MS spectrum of dehydrorabelomycin isolated from *S. lividans* LJ1018/8F5. **(D)** Structure of dehydrorabelomycin. **(E)** Quantification of the production of dehydrorabelomycin by 8F5-carrying exconjugants in five expression hosts on R3 agar medium.

produced by the original host SBT5/8F5. The highest yield of dehydrorabelomycin was produced by *S. lividans* LJ1018/8F5, reaching a level of 118.0 mg/L (**Figure 6E**).

Discovery of an Actinomycin D BGC Using LEXAS and *S. lividans* GX28

Streptomyces parvulus 10 was isolated from the marine sponge *Carteriospongia foliascens* collected from the South China Sea by the Zhiyong Li Group. We constructed a cosmid library of the *S. parvulus* 10 genome and used *S. lividans* GX28 as the heterologous expression host for high-throughput library screening. An exconjugant exhibiting *S. aureus* inhibitory activity was observed. Sequencing analysis of the corresponding cosmid, 5H11, revealed that it contains an NRPS BGC (**Figure 7A**) with 16 genes highly similar (78–95% similarity) to genes of the actinomycin C of *Streptomyces anulatus* (Keller et al., 2010). The compound produced by *S. lividans* GX28/5H11 was determined to be actinomycin D (**Figure 7B**) by HPLC and LC-MS (*m/z* of $[M+H]^+$ obsd. 1255.6359, calcd. 1255.6363) (**Figures 7C,D**).

To compare the heterologous expression of actinomycin BGC in different hosts, 5H11 was transferred to the five *S. lividans* hosts, and the exconjugants were fermented in YBP medium. After 4 days of fermentation, the bioactivity test indicated that the inhibition zones produced by the exconjugants of the newly engineered *S. lividans* hosts were larger than for the parental strain. The *S. lividans* LJ1018/5H11 fermented culture displayed the largest inhibition zone against *B. mycoides* (**Figure 7E**). HPLC quantitative determination confirmed that the production of actinomycin D in *S. lividans* strains increased in turn ($p < 0.01$ or $p < 0.001$), i.e., the production in LJ1018/5H11 > GX28/5H11 > GX27/5H11 > GX25/5H11 > SBT5/5H11 (**Figure 7F**). LJ1018/5H11 was capable of producing 52.1 mg/L actinomycin D, which was 3.5 times the level produced by SBT5/5H11. The native strain *S. parvulus* 10 also synthesized actinomycin D (18.0 mg/L). The actinomycin D yields of strains GX27/5H11, GX28/5H11, and LJ1018/5H11 were 32% ($p < 0.0001$), 95% ($p < 0.0001$), and 190% ($p < 0.0001$) higher than that of *S. parvulus* 10, respectively.

DISCUSSION

Whether a host can effectively express all the essential genes of a given heterologous synthetic pathway is key to the success of the heterologous production of secondary metabolites. When

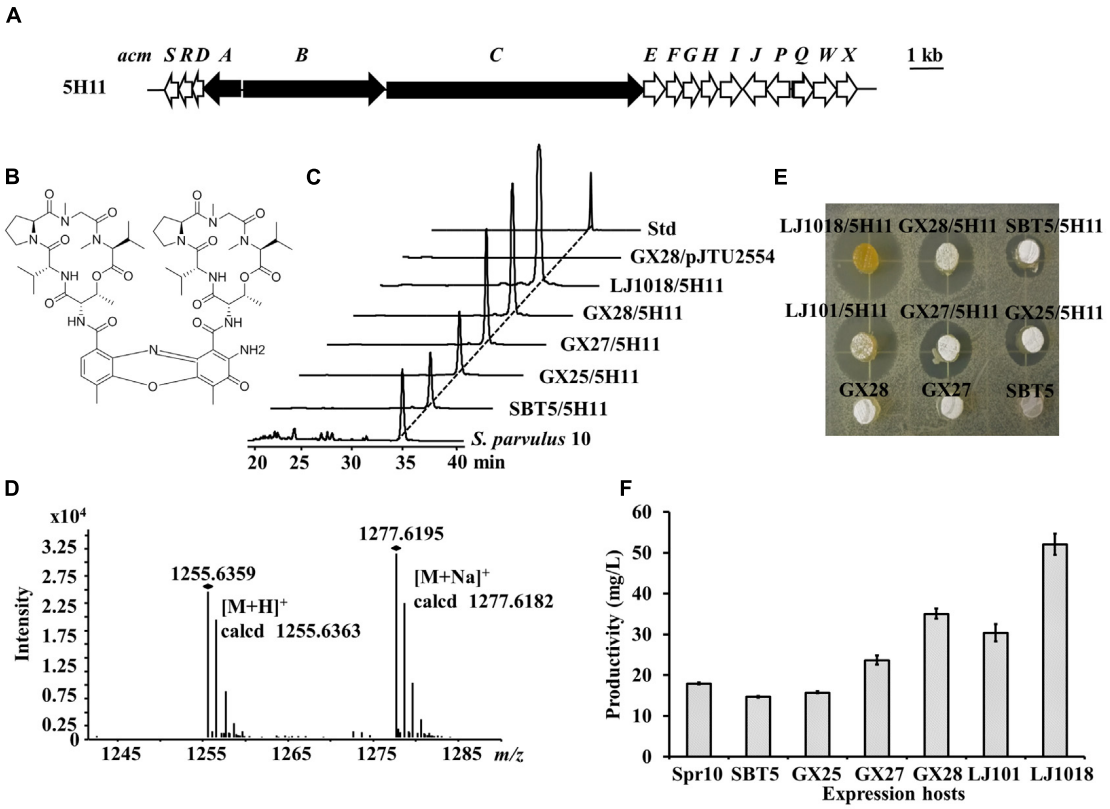

FIGURE 7 | Identification of actinomycin D and its BGC by LEXAS screening of the *Streptomyces parvulus* 10 genomic cosmid library using *S. lividans* GX28 as host. **(A)** Gene organization of the actinomycin D BGC in cosmid 5H11. The PKS genes are black. **(B)** Structure of actinomycin D. **(C)** HPLC analysis of the extracts of fermented cultures of *S. lividans* exconjugants carrying 5H11. The absorbance was measured at 440 nm. Std, actinomycin D standard. **(D)** HR-MS spectrum of actinomycin D isolated from *S. lividans* GX28/5H11. **(E)** Bioassay against *B. mycoides* to detect actinomycin D production by 5H11 exconjugants. Agar plugs of fermented cultures of 5H11-containing *S. lividans* exconjugants were placed on LB agar pre-spread with *B. mycoides*. The plates were incubated for 12 h at 37°C for observing zones of inhibition. **(F)** Quantification of the production of actinomycin D by *S. parvulus* 10 and the 5H11-carrying exconjugants in six expression hosts on R3 agar medium. Spr10, *S. parvulus* 10.

high-throughput heterologous expression methods are used to screen metagenomic or genomic libraries (Baltz, 2008), ideally overall gene expression should be improved by manipulating global regulatory genes in the expression host, rather than by attempting to modify all of the individual promoters within BGCs, a potentially complex and cumbersome task (Chen et al., 2010) and one not possible with previously unknown BGCs. Although previously engineered hosts, such as *S. coelicolor*, have altered global regulatory genes to promote the expression of BGCs, due to the restriction of methylated DNA and the slightly lower frequency of conjugative transfer (MacNeil, 1988), these strains are not well-suited to be LEXAS high-throughput screening hosts (Chen et al., 2012).

Streptomyces lividans has the advantage of high frequency of conjugative transfer and no restriction on exogenous methylated DNA (Martinez et al., 2004), and *S. lividans* TK24 strain itself contains an *rpsL*[K88E] mutation that promotes gene expression (Ochi, 2007). We previously added 1–2 copies of the global regulatory gene $afsRS_{cla}$ to the TK24 genome, and the resultant strains indeed contributed significantly to the establishment of a high-throughput library expression and screening system (LEXAS) (Xu et al., 2016). To further optimize the *S. lividans* host and the LEXAS system, in this study we continued to optimize *S. lividans* with global regulatory genes, including $nusG_{sc}$ and $wblA_{sl}$, as well as drug efflux pump genes, in addition to $afsRS_{cla}$.

Many antibiotic BGCs carry export genes, such as *actII-ORF2* in the actinorhodin BGC (Fernándezmoreno et al., 1991) and *rifP* in the rifamycin BGC (August et al., 1998). These efflux pumps secrete the antibiotics out of the cell, thereby reducing the feedback inhibition of the end-products on the biosynthetic enzymes, while increasing the self-tolerance to the antibiotics. Therefore, overexpression of antibiotic efflux pumps is helpful when engineering strains to increase the production of antibiotics of interest (Qiu et al., 2011). MdfA of *E. coli* is a multi-drug transporter of the major facilitator superfamily (Sigal et al., 2006); it has a broad-spectrum recognition and efflux function for toxic compounds and enhances the tolerance of the host strains to natural or synthetic antibiotics such as daunomycin, rifampin, puromycin, aminoglycoside antibiotics, and quinolones (Edgar and Bibi, 1997). LmrA of *Lactococcus lactis* subsp. *cremoris* MG136362 belongs to a family of multidrug resistance ABC (ATP-binding cassette) transporters driven

by ATP hydrolysis (van Veen et al., 1996; Wilkens, 2015), and its sequence is highly similar to that of the multi-drug resistance export pump P-glycoprotein (MDR) in mammals (Margolles et al., 1999). LmrA and MDR1 increased the tolerance of bacterial cells to compounds such as daunomycin, ethidium, rhodamine 6G, and tetraphenylphosphonium (van Veen et al., 1996). We added two codon-optimized efflux pump-encoding genes, $mdfA_{co}$ and $lrmA_{co}$, into *S. lividans* GX25 to construct GX27. Our quantitative data on heterologous expression suggested that the introduction of these two efflux pump genes significantly increased the yield of four antibiotics, including actinorhodin, dehydrorabelomycin, piericidin A1, and actinomycin D, demonstrating that it is applicable to use multi-drug transporters for the general improvement of antibiotics production in heterologous hosts.

The second group of ideal engineering targets are global regulators. NusG, the regulator of the NusG-like family, functions as an RNAP processivity clamp and is the only anti-terminator factor conserved among the kingdoms of prokaryotes, eukaryotes, and archaea (Burmann et al., 2010). Behnken et al. (2012) used the constitutive strong promoter *Pthl* to increase the expression level of *nusG*, thereby successfully activating the originally silenced polythioamides BGC in the genome of the anaerobic bacterium *Clostridium cellulolyticum* and unveiling seven new compounds. In this study, we inserted *S. coelicolor* $nusG_{sc}$ into the *S. lividans* SBT5 and used the resultant GX25 as a heterologous host to express six different types of antibiotic BGCs, the production of five out of six antibiotics increased significantly ($p < 0.05$). Similarly, additional copy of the positive regulatory gene $afsR_{cla}$ and sigma factor-like gene $afsS_{cla}$ increased the production of six antibiotics significantly. These results suggest that global positive regulatory genes like $nusG_{sc}$ and $afsRS_{cla}$ are applicable for improving the heterologous expression of PKS, NRPS, and NRPS-PKS BGCs.

WblA is a global negative regulator unique to actinomycetes (Kang et al., 2007) and has obvious sequence similarity to the developmental differentiation factor WhiB (Chater et al., 2000). In many actinomycetes, knocking out *wblA* significantly improved antibiotic biosynthesis in the mutant strains (Kang et al., 2007; Noh et al., 2010; Rabyk et al., 2011; Nah et al., 2012; Yu et al., 2014). The molecular mechanism by which WblA negatively regulates antibiotic synthesis remains unclear. We knocked out $wblA_{sl}$ in *S. lividans* GX28 to obtain LJ1018, which led to significant increases in the production of hybrubins, dehydrorabelomycins, and actinomycin D ($p < 0.05$), and slight increases of the three antibiotics actinorhodin, murayaquinone, and piericidin A. However, the mutant strains *S. lividans* LJ1018 and LJ101 that we constructed do not produce spores. This is not surprised since WblA plays an important role in the formation of aerial hyphae in *Streptomyces* (Fowler-Goldsworthy et al., 2011). After knocking out *wblA* in *S. coelicolor* and *S. chattanoogensis* L10, no spores were formed on the aerial hyphae (Yu et al., 2014). As a consequence, we had to use mycelium instead of spores as the recipient during conjugation transfer, which reduced the frequency of conjugation sharply. Nevertheless, this characteristic did not affect the introduction of target BGCs into the host for heterologous expression, since dozens to 100s of exconjugants could be obtained for each mycelium conjugation in our laboratory. However, when we attempted to use *S. lividans* LJ1018 mycelium as LEXAS host for high-throughput expression of arrayed cosmid libraries and BAC libraries, only sporadic exconjugants emerged, so LJ1018 is not suitable as a host for high-throughput heterologous expression of arrayed libraries.

In contrast, strains GX25, GX27, and GX28 still produce abundant spores. Both high-throughput and conventional conjugation transfer worked as efficiently as with the parental strain SBT5. When screening the two cosmid libraries (from *S. galtieri* Sag48 and *S. parvulus* 10) using GX28 as the high-throughput expression host, 3948 out of 4032 cosmid clones (98%) yielded exconjugants, and when we screened a BAC library using GX28, 818 out of the 912 clones (93%) produced exconjugants. BGCs producing dehydrorabelomycin and actinomycin were identified from the cosmid libraries. Five clones containing the complete piericidin A1 BGC, in addition to clones carrying the murayaquinone BGC, were identified from the *S. griseoruber* Sgr29 genomic BAC library. Notably, these piericidin BGC clones had been overlooked during the previous screening using SBT5 as a host (Gao et al., 2017). Indeed, the corresponding SBT5 exconjugants did not show significant antibacterial activity, since no zone of inhibition was produced. Our genomic screening results demonstrate that the GX28 strain is an excellent expression host for the LEXAS procedure to screen for functional BGCs in arrayed cosmid libraries and BAC libraries, and also demonstrates the superiority of GX28 as a heterologous expression host.

In summary, *S. lividans* provides excellent host strains for high-throughput screening of genomes (such as LEXAS screening) due to its rapid growth, abundant sporulation, high frequency of conjugation transfer, no methylation restriction on methylated DNA, and efficient expression of heterologous BGCs after rational engineering. By sequentially engineering global regulatory genes and multi-drug transporters, we obtained four engineered strains of *S. lividans*, which in turn increased the yield of multiple synthesized antibiotics that involve PKS, NRPS, and PKS-NRPS hybrid pathways. Since cryptic BGCs in microorganisms usually encode new or unknown biosynthetic pathways, it is very difficult to specifically engineer pathway-specific regulatory factors or to appropriately modify promoters. Our optimized host GX28 produces high yields of antibiotics and does not require one-by-one modification of the promoters in a BGC of interest, so it is an excellent heterologous expression host for LEXAS high-throughput screening of cosmid or BAC libraries for the discovery of new, previously silenced BGCs and corresponding compounds. In addition, our study has revealed that the positive regulatory genes $nusG_{sc}$ and $afsRS_{cla}$, the negative regulatory gene $wblA_{sl}$, and efflux pump genes, which are not regulatory genes by definition, have synergistic effects on the synthesis of antibiotics when they are combined in one host. The yields of the tested antibiotics were increased several times, even dozens of times in the case of hybrubins, over yields from the parent strain, SBT5. Furthermore, since the strain engineering conducted here mainly utilizes plasmid integration, these plasmids, especially pJTU6728, which integrates *nusG*,

afsRS$_{cla}$, and efflux pump genes, can be used for the engineering of other strains for high-yield production of antibiotics in future. Therefore, the strains we have generated and the approaches we have used should aid in the identification of new BGCs and in optimizing the production of secondary metabolites of clinical and industrial value.

AUTHOR CONTRIBUTIONS

MT, ZD, and ZL were responsible for the original concept and designed the experiments. MT and YW analyzed the data. QP, GG, JL, QL, XC, FZ, MX, KL, and YW performed the experimental work. QP and MT wrote the manuscript. All authors read and approved the final manuscript.

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REVIEW
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Exploring Structural Diversity of Microbe Secondary Metabolites Using OSMAC Strategy: A Literature Review

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Microbial secondary metabolites (MSMs) have played and continue to play a highly significant role in the drug discovery and development process. Genetically, MSM chemical structures are biologically synthesized by microbial gene clusters. Recently, however, the speed of new bioactive MSM discovery has been slowing down due to consistent employment of conventional cultivation and isolation procedure. In order to alleviate this challenge, a number of new approaches have been developed. The strategy of one strain many compounds (OSMAC) has been shown as a simple and powerful tool that can activate many silent biogenetic gene clusters in microorganisms to make more natural products. This review highlights important and successful examples using OSMAC approaches, which covers changing medium composition and cultivation status, co-cultivation with other strain(s), adding enzyme inhibitor(s) and MSM biosynthetic precursor(s). Available evidences had shown that variation of cultivation condition is the most effective way to produce more MSMs and facilitate the discovery of new therapeutic agents.

Keywords: OSMAC strategy, microbe secondary metabolite, structural diversity, medium composition, co-cultivation, epigenetic modification

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INTRODUCTION

Microbial secondary metabolites (MSMs) have been recognized as the primary source of new compounds for drug discovery and development (Gunatilaka, 2006; Rateb et al., 2011b; Deng et al., 2013). Traditional chemical investigation of microorganism mainly focuses on extraction and isolation of structurally and highly active compounds from fermentation broth and mycelium. However, these processes are becoming inefficient due to high rate of the re-discovery of known MSMs. It is commonly believed that a large portion of microbial gene clusters are silenced under standard fermentation conditions (Scherlach and Hertweck, 2009; Wasil et al., 2013). By mining microbial genome and targeting biosynthetic gene clusters of MSM, researchers can exploit the potential of microbes in a more objective way, such as knocking down, introduction or heterologous expression of microbial genes, regulation of promoters, induction of mutations, or changing cultivation conditions to stimulate MSM genes expression (Schneider et al., 2008). Variation of

cultivation condition has been deemed to be the simplest and most effective strategy, which is termed as "one strain many compounds (OSMAC)" by professor Zeeck and co-workers (Bode et al., 2002). On basis of extensive literature search, important and successful examples using OSMAC strategy are summarized in this review, which consists of variation of medium, changing cultivation condition, co-cultivation with other strain(s), adding epigenetic modifier(s) or biosynthetic precursor(s).

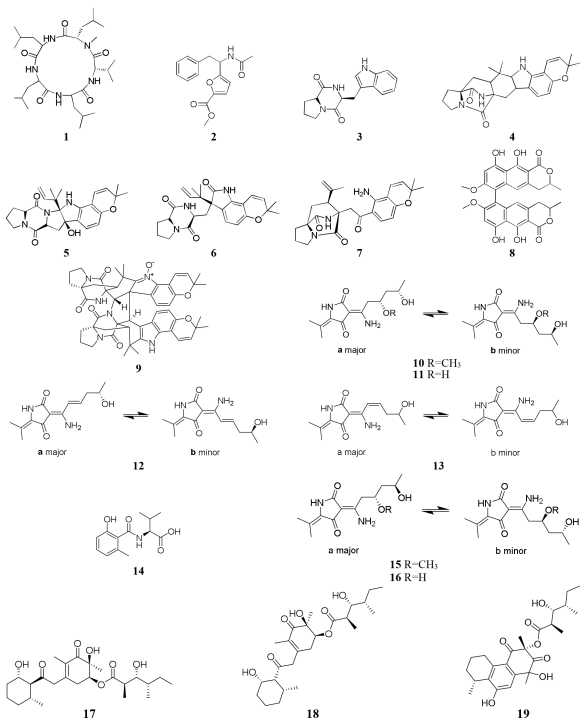
VARIATION OF MEDIUM

Culture medium has a greater effect not only on microbe growth but also on metabolism. It has been reported that C/N ratio, salinity, and metal ion can regulate the degree and pattern of MSM gene expression and result in production of various secondary metabolites.

Medium Composition

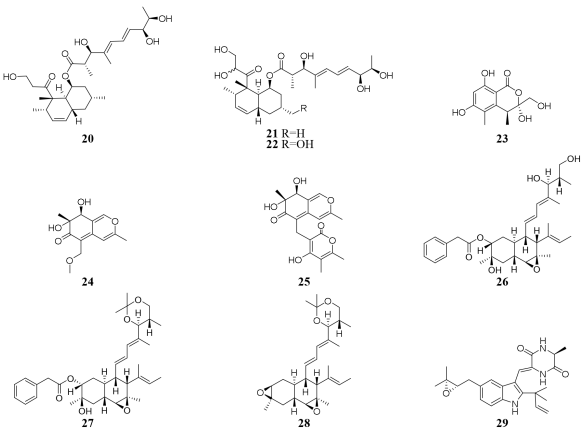
Generally, carbon and nitrogen sources are major components in the culture medium. The carbon source not only provides the basis for building biomass and represents the source of energy for all heterotrophs but also delivers carbon units for secondary metabolites. The nitrogen source is required for the synthesis of essential proteins and nucleic acids, and likewise *N*-containing units for secondary metabolites. The type of used carbon and nitrogen sources is known to have a significant influence on microbial secondary metabolism (Ruiz et al., 2009; Singh et al., 2017). Furthermore, the C/N ratio is one of important factors that affect fermentation products (Karakoç and Aksöz, 2004; Brzonkalik et al., 2012; Dinarvand et al., 2013). Notably, the consumption of carbon and nitrogen-based medium components can greatly affects the pH of the cultivation broth, e.g., by formation of organic acids or the accumulation of basic ammonium. Thus, microorganisms cultured in medium containing different components may exhibit differently adapted metabolism and express specific sets of biosynthetic genes, which produced a differential biosynthesis of specialized metabolites (Ma et al., 2009).

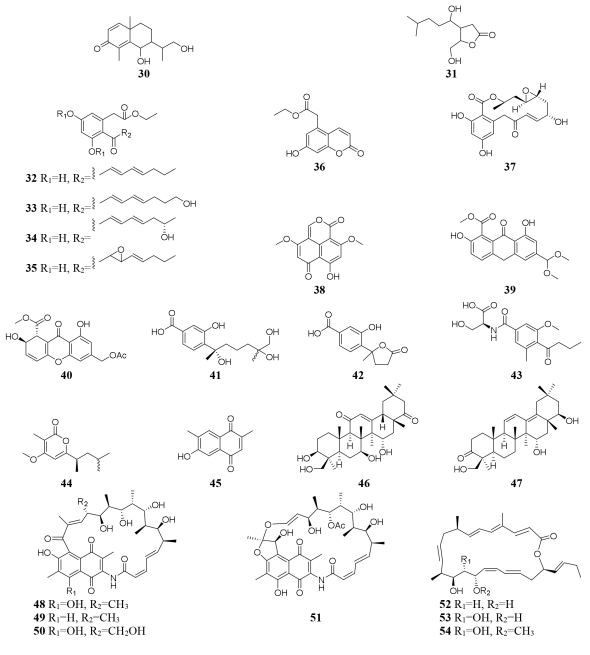
One marine-derived strain *Asteromyces cruciatus* 763 was shown to produce a new pentapeptide lajollamide A (**1**), when cultivated in the Czapek-Dox broth contained arginine solely as nitrogen source rather than $NaNO_3$, which was missed in the normal Czapek-Dox medium (Gulder et al., 2012). One sediment-derived *Aspergillus niger* BRF-074 produced a novel furan ester derivative (**2**), a compound has toxicity acidity against HCT-116 cancer cell line (Uchoa et al., 2017), when cultivated in MPDB (malt peptone dextrose broth) medium. But this compound failed to appear in PDB (potato dextrose broth) or PDYB (potato dextrose yeast broth) media. A fungus *Aspergillus* sp. from Waikiki Beach (Honolulu, HI), generated six isotopically labeled metabolites (**3**–**8**) when grown on the deuterium-enriched Czapek broth (Wang et al., 2015a), whereas this strain was found to metabolite a novel prenylated indole alkaloid, waikialoid A (**9**) when cultivated in PDB medium. Bioassay results indicated that compound **9** possessed potent inhibitory effect on biofilm formation of *Candida albicans* with an IC_{50} value of 1.4 μM (Wang Q.X. et al., 2012).



Five new polyketides (**10**–**14**) were detected in the crude extract of rice-based medium of a marine-derived *Cladosporium sphaerospermum* 2005-01-E3 (Wu et al., 2014). Another two new hybrid polyketides (**15**–**16**) were accessed when the same strain was fermented on the soybean flour (Yu et al., 2015). The organic extract of *Dothideomycete* sp. CRI7 was elaborated by four comparative medium. The strain growing in PDB made with potato tubers led to the isolation of azaphilone derivatives (**17**–**18**) and a novel tricyclic polyketide (**19**). Only compound **19** exhibited a broad spectrum of cytotoxic activities (Senadeera et al., 2012). It is interesting that MSM production by strain CR17 was sensitive to sources of potato and malt extract used for the preparation of PDB and Czapek malt media, respectively. Three new polyketides (**20**–**22**) were produced when strain CR17 was grown in PDB broth prepared from a commercial potato powder instead of fresh tubers of potato, while this strain produced several other compounds (**20**–**21** and **23**–**25**) in Czapek malt medium. Compound **24** exhibited cytotoxic activity against cancer cell lines MOLT-3, HuCCA-1and A549 with IC_{50} values of 17.4, 48.1, 46.5 μg/mL, respectively (Hewage et al., 2014). One fungus strain of *Fusarium tricinctum* isolated in Beni-Mellal, which can colonize the rhizomes of *Aristolochia paucinervis*, could afforded three new fusarielins (**26**–**28**). But these metabolites were not detected when cultivated in normal rice medium supplemented with fruit and vegetable juice. Bioassay results suggested that compound **26** possessed cytotoxic effect on human ovarian cancer cell line A2780 with an IC_{50} value of 12.5 μM (Hemphill et al., 2017). A new diketopiperazine (**29**) was isolated from *Eurotium rubrum* MPUC136 cultured by

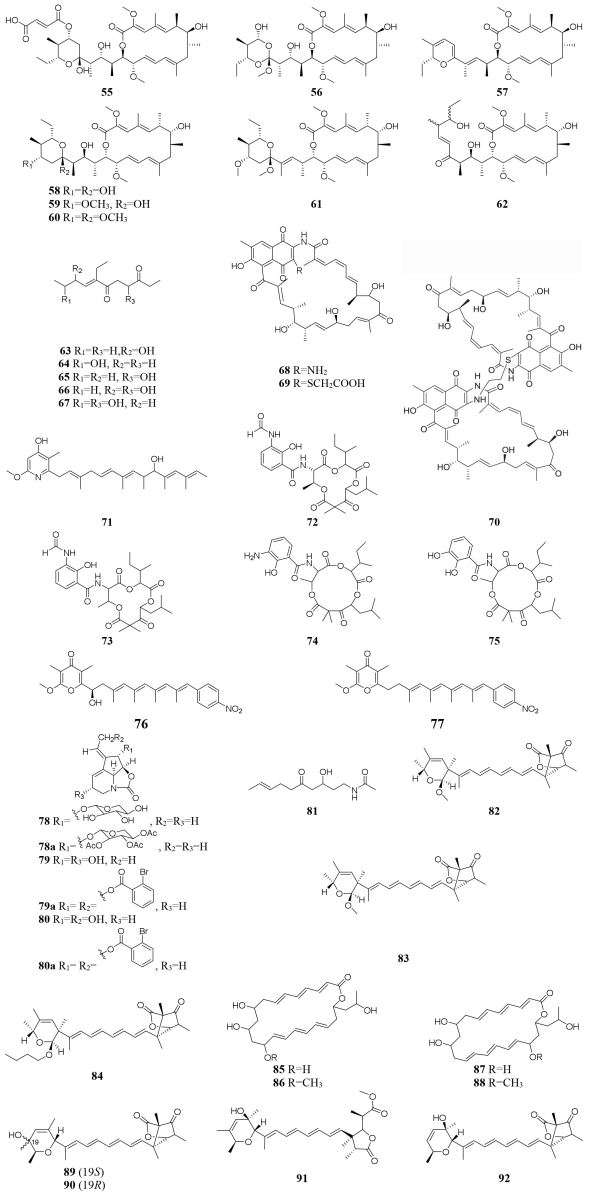
wheat medium, which displayed more powerful bioactivity than the Czapek-Dox agar medium, and shown to have cytotoxicity against B_{16} melanoma cell line with an IC_{50} value of 60 μM (Kamauchi et al., 2016).





HPLC analysis of crude extracts of an actinomycete strain *Lentzea violacea* AS08 indicated different composition in three media including CYPS (casein yeast peptone), SCP-1 (starch casein peptone), and SC (starch casein) (Hussain et al., 2017). Only one new eudesmane sesquiterpenoid (**30**) and a new analog of virginiae butanolide E (**31**) were detected in SC medium, and compound **30** exhibited moderate cytotoxic effect on HCT-116 and A549 tumor cell lines with IC_{50} values of 19.2 and 22.3 μM, respectively. One rhizosphere fungus *Paraphaeosphaeria quadriseptata* produced a known C18 polyketide monocillin I together with several analogs when incubated in PDA medium constituted with tap water (Wijeratne et al., 2004). However, the same fungal strain could make six new trihydroxybenzene lactones, cytosporones F-I (**32**–**37**), when the tap water was changed as distilled water (Paranagama et al., 2007). Similarly, one new naphthalopyran compound (**38**), which possesses an unusual oxygenated aromatic structure with a lactone bridge, could be metabolized by the fungus *P. hordei* grown on plant tissue agar such as macerated tulip and yellow onion, oatmeal and red onion, while it was not detected in CYA (caffeic acid agar), MEA (malt extract agar), and YES (yeast extract with supplements) media (Overy et al., 2005). When cultivated in rice medium, a hard coral-derived fungus *Scopulariopsis* sp. from the coastline of Red Sea was shown to afford six secondary metabolites including xanthone derivatives (**39**–**40**), phenolic bisabolane-type sesquiterpenes (**41**–**42**), one new alkaloid (**43**) and one new α-pyrone derivative (**44**) (Elnaggar et al., 2016). Interestingly, this strain could biosynthesize a new naphthoquinone derivative (**45**) and two new triterpenoids (**46**–**47**) in the protein-rich white bean medium (Elnaggar et al., 2017).

Chemical investigation of one marine-derived strain *Streptomyces* sp. C34 grown on ISP2 (yeast malt extract agar) medium led to the isolation of four new ansamycin-type polyketides (**48**–**49**). But only compounds **48**, **50,** and **51** could be extracted from modified ISP2 medium, which contained glycerol rather than glucose. Bioassay results indicated that compound **51** had a selective inhibitory effect on *S. aureus* ATCC 25923 with a MIC value of 0.05 μg/mL (Rateb et al., 2011a). The utilization of a defined medium to cultivate strain C34 resulted in the observation of three novel 22-membered lactone polyketides (**52**–**54**) (Reid et al., 1995). Compounds **50**–**52** possessed strong antibacterial activities against *L. monocytogenes* and *B. subtilis* with MIC values range from 3 to 6 μg/mL and against *S. aureus* with MIC values of <1 μg/mL (Rateb et al., 2011a). Four media applied to strain *Streptomyces* sp. CS resulted in production of various natural products including three new macrolides (**55**–**57**) from YMG agar medium, five new 16-membered macrolides (**58**–**62**) from ISP2 broth, five novel polyketides (**63**–**67**) from sterilized Waksman Synthetic medium and three new naphthomycins (**68**–**70**) from oatmeal medium. Compounds **55** was shown to have inhibitory effect on *Fusarium moniliforme* with a MIC value of 300 μg/mL and compounds **58**–**62** exhibited cytotoxicity toward the MDA-MB-435 human cancer cell line with IC_{50} values of 4.2, 4.5, 5.5, 3.8, and 11.4 mM, respectively (Lu and Shen, 2003, 2004; Li et al., 2008, 2010; Yang et al., 2012). *Streptomyces* sp. ML55 in a medium consisting of glycerin, molasses, casein, polypeptone led to the isolation of three novel antimycins, JBIR-02 (**71**), JBIR-06 (**72**), and JBIR-52 (**73**), while this strain had capacity to produce two novel depsipeptides (**74**–**75**) in GYM medium (Ueda et al., 2007, 2008; Kozone et al., 2009; Li X. et al., 2013). An ant-derived actinomycete *Streptomyces* sp. 1H-GS5 was found to produce one new spectinabilin derivative (**76**) when cultivated in the medium consisting of corn starch 10%, soybean powder 1%, cotton flour 1%, α-amylase 0.02%, NaCl 0.1%, K_2HPO_4 0.2%, $MgSO4 \cdot 7H_2O$ 0.1%, $CaCO_3$ 0.7%, cyclohexanecarboxylic acid 0.1%, pH 7.0, while this stain made another new cytotoxic spectinabilin (**77**) when reducing the proportion of nutrients (Liu S. et al., 2015; Liu C. X. et al., 2016).



When cultured in an oat bran medium, one strain *Streptomyces* sp. A1 was found to produce rubromycin derivatives, while other three known compounds were biosynthesized in a mannitol/soybean meal medium and three new congeners (**78**–**80**) and streptenol E (**81**) in medium (degreased soybean meal 2%, mannitol 2%, agar 2%) with soil as an addition, provide. Compound **81** had significant cytostatic effect on four tumor cell lines including HMO2, HEP G2, MCF7 and Kato III with GI_{50} values (the concentration that causes 50% growth inhibition) of 0.15, 0.3, 10, and 0.7 μM, respectively (Puder et al., 2001). Phytochemical study of one filamentous soil fungus, *Talaromyces wortmannii*, cultivated in maize culture medium, led to the separation of three new polyketones (**82**–**84**), which were absent in rice or dextrose agar media. Compounds **82**–**84** displayed inhibitory activities against NFRD (fumarate reductase) with IC_{50} values of 8.8, 11, and 13 μM, respectively (Liu W. C. et al., 2016). Interestingly, this stain was found to produce four novel 22-membered macrolides (**85**–**88**) (Dong et al., 2006) and four novel tetraene lactones (**89**–**92**) (Dong et al., 2009) when grown in the still-cultured medium (2.5% soybean meal and 97.5% rice). Compounds **85**–**88** exhibited *in vitro* moderate cytotoxic activities against human cancer cell lines (HCT-5, HCT115, A549, MDA-MB-231, and K562) with IC_{50} values range from 28.7 to 130.5 μM, while compounds **89**–**92** showed potent inhibitory effects on cathepsin B.

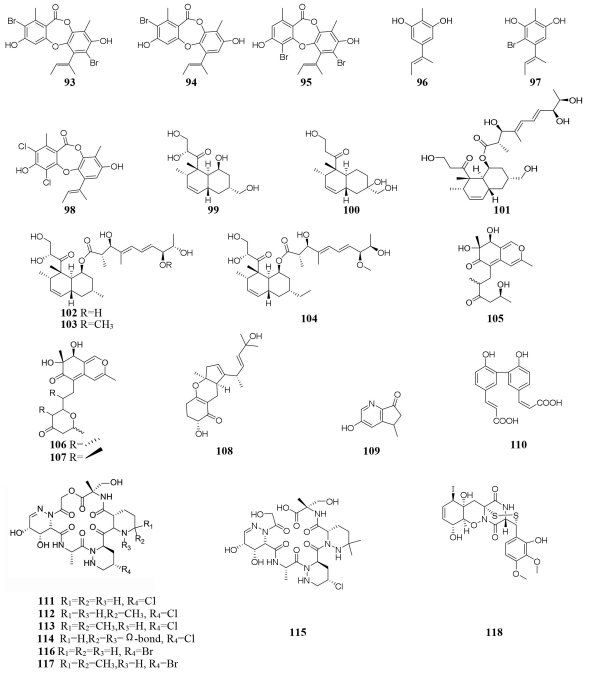
Salinity

Salinity is an important factor in determining many aspects of the chemistry of natural water and of biochemical process within cultivation system, and is a thermodynamic state variable that, along with temperature and pressure, governs physical characteristics like the osmotic pressure and enzymes involved in microbial growth and metabolism (Blunt et al., 2015). Suitable salinity is needed for normal microbial growth and high osmotic pressure makes cells dehydrated and affects microbial biochemical reactions (Poolman and Glaasker, 1998; Wang Y. et al., 2011).

Microorganisms exposed to different types of media supplemented with various halogens maybe trigger their synthesis pathway to restore osmotic imbalance, thus activating different hidden MSM biosynthetic gene clusters. Compare to that grown in seawater, one marine-derived fungus *Aspergillus unguis* CRI282-03 was shown to produce new brominated depsidones (**93**–**95**) and two new orcinol derivatives (**96**–**97**) in KBr medium and a new depsidone (**98**) in KI broth (Sureram et al., 2013). Bioassay results indicated that compounds **95** and **96** possesses aromatase inhibitory effects (Sureram et al., 2012). Nine new polyketides (**99**–**107**), which were absent in the broth contained KI or deionized water, were produced by the fungus *Dothideomycete* sp. CRI7 isolated from *Tiliacora triandra* when cultivated in the medium supplemented with KBr and seawater (Wijesekera et al., 2017).

Chemical investigation of one symbiotic stain *Aspergillus* sp. D from *Edgeworthia chrysantha* led to isolation of five known heterocyclic alkaloids from normal Czapek medium, while a new meroterpenoid (**108**) and four known analogs were obtained from Czapek medium with 3% salty (Zhang et al., 2018a,b). One mangrove-derived endophyte *Wallemia sebi* PXP-89 cultivated in 10% NaCl broth produced a new cyclopentanol pyridine alkaloid (**109**), which was not detected in normal medium (Peng et al., 2011). When cultivated in medium containing 10% sea salt, strain *Spicaria elegans* KLA-03 was shown to biosynthesize a new antimicrobial diacrylic acid (**110**) (Wang F. Z. et al., 2011). Strain *Streptomyces* sp. DSM 14386 could metabolize five new compounds (**111**–**115**) in 1.5% NaCl medium, while this strain produced two brominated congeners (**116**–**117**) in 1.5% NaBr medium. Antimicrobial tests showed that compounds **113** and **117** displayed potent antibiotics against MRSA (methicillin-resistant *Staphylococcus aureus*) with the same MIC values of 16 μg/mL, and compound **117** also had strong activity toward *Mycobacterium smegmatis* (IC_{80} = 2 μg/mL) (Onaka, 2017). Two rare epidithiodiketopiperazines, gliovirin and pretrichodermamide A, were detected in 1.5% NaCl

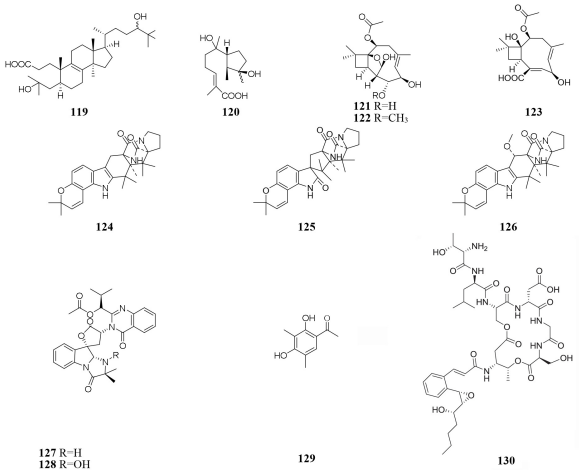
broth of a marine-derived *Trichoderma* sp. TPU199, while this strain produced a new iodo derivative (**118**) from freshwater medium with 3.0% NaI and 3.0% NaBr as well as 5-bromo-5-deoxy derivative (Yamazaki et al., 2015a).



Metal Ion

Metal ion affects physiological structure and function of microorganism. The interaction between metal ion and microbe is usually assumed in three pathways, including causing reactions in cells, conserving energy in the process of dissimilation, and assimilating reactions (Thorneley, 1990).

One marine-derived strain *Ascotricha* sp. ZJ-M-5 was shown to produce a new 3,4-split ring lanolin alkyl triterpene (**119**) and a new cyclonerols derivative (**120**), when cultivated in eutrophic medium made up with sea salt (Xie et al., 2013a,b). However, three new caryophyllene derivatives (**121**–**123**) were detected in modified Czapek Dox medium, while compound **122** was absent in the fermentation broth without Mg^{2+} (Wang W.J. et al., 2014). Strain *Aspergillus sclerotiorum* C10WU derived from hydrothermal vent sediment in Taiwan (China) could produce three new alkaloids (**124**–**126**) under normal medium. However, this strain metabolized one unelucidated compound due to the low amount available together with aspochracin when grown in the stressed culture medium with Cu^{2+} as a supplement. Likewise, two compounds, namely deoxytryptoquivaline and tryptoquivaline A (**127**–**128**), were purified from the normal extract of *A. clavatus* C2WU, while only metabolite **129** was found in normal medium containing Cu^{2+} and Cd^{3+} (Jiang et al., 2014). A novel antibacterial cyclodepsipeptide, named NC-1 (**130**), was produced by a red soil-derived strain *Streptomyces* sp. FXJ1.172 when cultured in GYM (glucose-yeast extract-malt extract) medium added with ferric ion (Liu M. et al., 2016).



CULTIVATION CONDITION

Suitable cultivation conditions, such as appropriate temperature, pH, oxygen concentration, and cultivation status, are essential for the growth and biochemical reactions of microorganisms. However, many biosynthetic genes of MSMs are not expressed under normal culture conditions, thus it is essential to change the cultivation condition to activate these silent gene clusters to diversify their MSMs.

Temperature

Chemical diversity of MSM is directly influenced by microbe enzyme activity, which is susceptible to cultivation temperature. The normal function of microbial enzyme is dependent on appropriate temperature. Generally, the higher the cultivation temperature is, the faster the enzyme deactivation rate will be (Feller et al., 1994). For example, when the temperature was lower than 30°C, secondary metabolites of an uncoded strain *Streptomyces* sp. were composed of chlortetracycline, while only tetracycline was synthesized when cultivation temperature went up to 35°C (Cui et al., 1996).

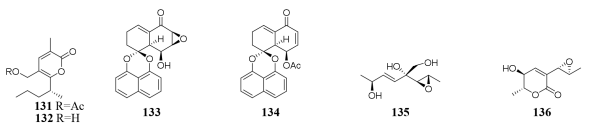
pH

During microbe fermentation process, the decomposition and utilization of nutrients as well as the accumulation of secondary metabolites usually causes the variation of medium pH (Gibson et al., 1988; Tan et al., 1998). It affects not only the activity of each enzyme, but also the surface charge of the membrane. The nature and permeability of cell membrane could change the rate of utilization of substrate, thus affecting the growth of microorganisms and biosynthesis of final products. Chemical study of one desert-derived strain *Nocardiopsis alkaliphila* nov. YIM-80379 led to isolation of two new pyran-2-one derivatives (**131**–**132**) when cultivated on Gause's synthetic agar slants with pH = 10. However, the neutral medium was unsuitable for its growth (Hozzein et al., 2004; Wang et al., 2013c). Acidic medium (pH = 5) dramatically increased the production of bioactive compounds of a mangrove-derived fungus *Rhytidhysteron rufulum* AS21B, including two

new antitumor spirobisnaphthalenes (**133**–**134**). However, these compounds were not detected in neutral medium (Siridechakorn et al., 2017).

Oxygen Concentration

Changes in oxygen supply can affect the biochemical reactions and activate different set of functional gene clusters for different secondary metabolites production (Sato, 1990). For example, [^{13}C]-labeled acetates and a small amount of [$^{18}O_2$] were used to investigate the biosynthetic pathway of aspinonene (**135**) in the culture broth of *Aspergillus ochraceus* DSM-7428. It is interesting that aspyrone (**136**) was produced by increasing dissolved oxygen concentration during fermentation, accompanied by reduced amounts of compound **135** under an oxygen enriched atmosphere (Fuchser et al., 1995).

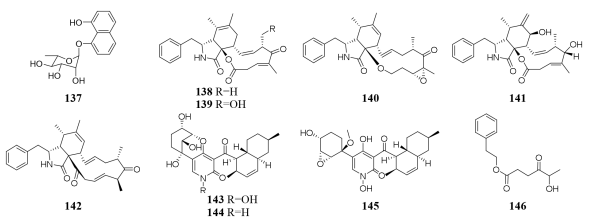


Cultivation Status

A growing body of evidence has indicated that cultivation status can directly affect microbe metabolic process, including solid or liquid, static or dynamic. Compared with solid and static cultivation, liquid and dynamic modes not only ensure the full contact of microorganisms and nutrients, but also affect their biochemical reactions by changing oxygen supply and activating functional gene clusters. Till now, MSMs from 12 genera had been investigated under different fermentation status, including *Arthrinium*, *Aspergillus*, *Myxotrichum*, *Nodulisporium*, *Lentinus*, *Paraphaeosphaeria*, *Penicillium*, *Pestalotiopsis*, *Phomopsis, Spicaria, Streptomyces, Ulocladium*.

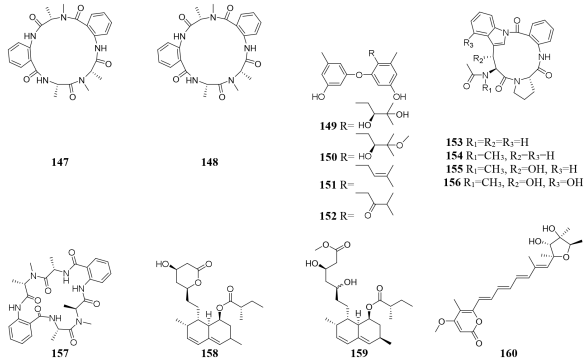
Arthrinium

One marine sponge-derived fungus *Arthrinium arundinis* ZSDS1-F was shown to metabolize a novel naphthalene glycoside (**137**) (Wang J.F. et al., 2014), five cytochalasins (**138**–**142**) (Wang et al., 2015b), and three alkaloids (**143**–**145**) when cultivated in a rotary liquid medium (Wang et al., 2015c). However, only phenethyl 5-hydroxy-4-oxohexanoate (**146**) was traced in rice medium (Li Y. L. et al., 2017). Bioassay suggested that compounds **143**–**146** possessed *in vitro* cytotoxicity against cancer cell lines A549, BGC823, Huh-7, K562, H1975, MCF-7, HL60, U937, Hela, and MOLT-4 with IC_{50} values in range of 0.24–45 μM. In addition, compounds **143** and **145** displayed significant AchE (acetylcholine esterase) inhibitory activity with IC_{50} values of 47 and 0.81 μM, respectively.



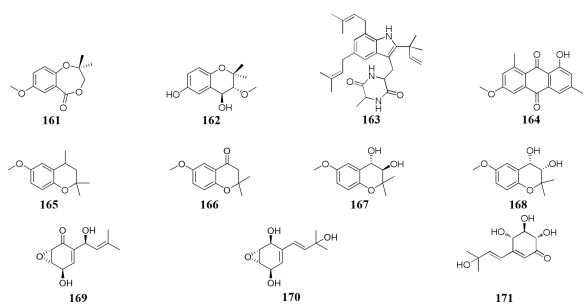
Aspergillus

By comparison of solid and liquid fermentation products of an endophytic strain *A. fumigates* LN-4 from stem bark of *Melia azedarach* L., their HPLC profiles were obviously different (Zhang et al., 2013). Strain *A. versicolor* ZLN-60 could produce two new cyclic pentapeptides (**147**–**148**) and four new prenylated diphenyl ethers (**149**–**152**) in static liquid condition (Zhou et al., 2011; Gao et al., 2013). Biological tests indicated that compound **151** displayed moderate cytotoxicity against Hela and K562 cancer cell lines with IC_{50} values of 31.5, 48.9 μM, respectively. However, further purification of its crude extract of solid medium resulted in the detection of four other novel cyclic peptides (**153**–**157**) (Peng et al., 2014). Chemical study of one marine-derived fungus *A. terreus* cultivated in 11 different culture conditions indicated that static agar was ideal for the production of antifungal lovastatins (**158**–**159**) and 7-desmethylcitreoviridin (**160**), which were absent in the shaking fermentation (Adpressa and Loesgen, 2016).



Lentinus

Two new prenyl phenols (**161**–**162**), one indole alkaloid echinuline (**163**) and one anthraquinone fiscione (**164**), were biosynthesized by *Lentinus strigellus* under static condition. While in shaking fermentation broth, this strain produced benzopyrans (**165**–**168**) together with panepoxydone (**169**) and isopanepoxydone (**170**). Bioassay indicated that striguellone A (**171**) displayed moderate cytotoxicity against HeLa cancer cells (Zheng et al., 2009; Barros et al., 2012).



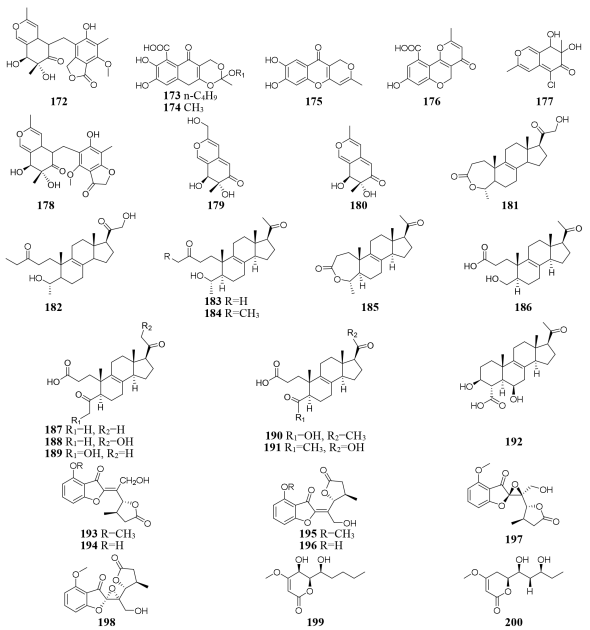
Myxotrichum

One fungal strain *Myxotrichum* sp. isolated from lichen *Cetraria islandica* (L.) Ach in Laojun Mountain (China), was shown to

make one novel austdiol analog (**172**), three new fulvic acid derivatives (**173**–**175**) and a new citromycetin analog (**176**) in rotary PDB medium (Yuan et al., 2013), while four new polyketides (**177**–**180**) were acquired from rice medium under static fermentation status. And compound **179** was shown to restrain Arabidopsis seeds root markedly with the inhibition rate of 75.9% at 8 μg/mL (Yuan et al., 2016).

Nodulisporium

Chemical investigation of one symbiotic strain *Nodulisporium* sp. (No. 65-12-7-1) from the lichen *Everniastrum* sp. resulted in the isolation of two rarely 4-methyl-progesteroids (**181**–**182**) when grown in rice medium (Zheng et al., 2013). Whereas this strain could biosynthesize ten novel nodulisporisteroids (**183**–**192**) in shaking PDB medium (Zhao et al., 2015).

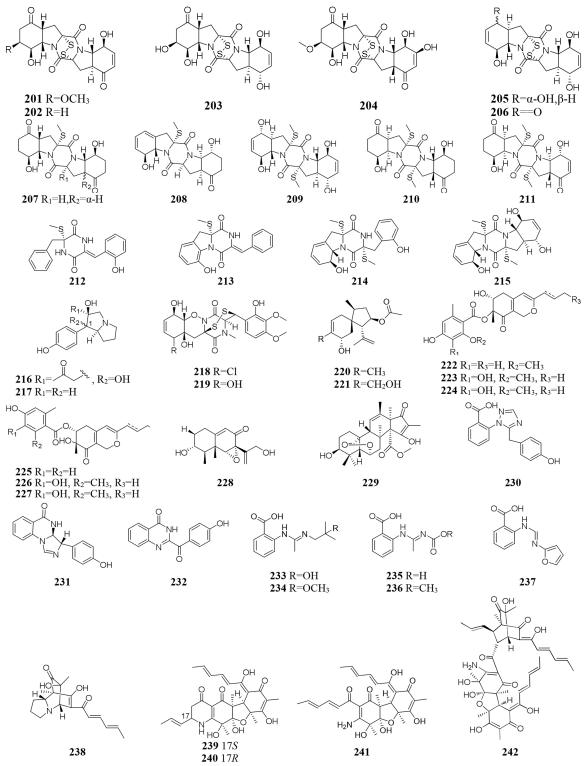


Paraphaeosphaeria

A fungal strain *Paraphaeosphaeria photiniae*, inhabiting *Roystonea regia* collected from Jianfeng Mountain (China), was shown to yield six new unique benzofuranone-derived γ-lactones (**193**–**198**) when cultivated in shaking liquid medium (Ding et al., 2009), while only two different δ-lactone derivatives (**199**–**200**) were detected in its rice medium (Ding et al., 2012).

Penicillium

When grown on solid PDA medium, one mangrove-derived fungus *Penicillium brocae* MA-231 could produce six new disulfide-bridged diketopiperazine derivatives (**201**–**206**). Bioassay results showed that compounds **201**, **202**, **205,** and **206** had cytotoxic activities against Du145, Hela, HepG2, MCF-7, NCI-H460, SGC-7901, SW1990, SW480, and U251 tumor cell lines with IC_{50} values ranging from 0.89 to 9.0 μM (Meng et al., 2014). When cultivated in liquid media (PDB or Czapek), however, five new penicibrocazines (**207**–**211**), four new thiodiketopiperazine alkaloids (**212**–**215**) and two new *N*-containing *p*-hydroxyphenopyrrozin derivatives (**216**–**217**) were detected in its fresh mycelia, which compounds **207**–**209** displayed antimicrobial activities against *Staphylococcus aureus* with MIC values of 32.0, 0.25, 8.0 μg/mL, respectively. In addition, **209**–**211** exhibited potent antimicrobial effect on *Gaeumannomyces graminis* with MIC values of 0.25, 8.0 and 0.25 μg/mL, respectively. And compound **216** showed powerful inhibitory effect on *Fusarium oxysporum* and *S. aureus* (Meng et al., 2015b, 2017).



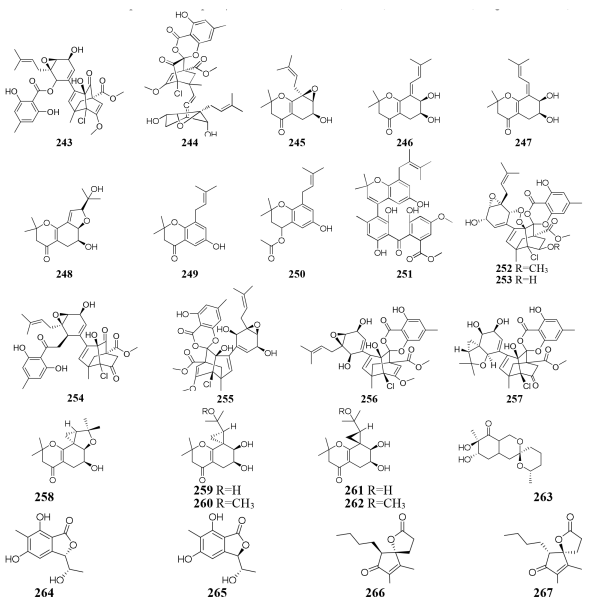
Chemical study of one marine sponge-derived strain *P. adametzioides* AS-53 led to isolation of two new bisthiodiketopiperazine derivatives (**218**–**219**) from shaking PDB broth, whereas two new acorane sesquiterpenes (**220**–**221**) were found in its static rice medium. Compound **218** showed strong lethality against brine shrimp (*Artemia salina*) with an LD_{50} value of 4.8 μM and a broad spectrum of antimicrobial effect on *Aeromonas hydrophilia*, *S. aureus*, *Vibrio* spp. *V. harveyi*, *Gaeumannomyces graminis* and *V. parahaemolyticus* (Liu Y. et al., 2015). Six novel azaphilone derivatives (**222**–**227**) as major secondary metabolites were obtained from rotary PDB medium of one marine-derived strain *P. commune* QSD-17 (Gao et al., 2011), whereas other new compounds isophomenone (**228**) and 3-deacetylcitreohybridonol (**229**) were detected in its static rice medium (Gao et al., 2012).

Three novel penipanoids (**230**–**232**) were characterized from one marine-derived strain *P. paneum* SD-44 grown in rice medium (Li et al., 2011). The exploration of changing fermentation conditions of *P. paneum* SD-44 to a seawater-based culture broth under dynamic fermentation condition gave five

new anthranilic acid derivatives (**233**–**237**). Metabolites **233** and **237** exhibited inhibitory activity toward human colon cancer RKO cell lines with IC_{50} values of 8.4, 9.7 μM, respectively (Li C. S. et al., 2013). One deep sea-derived fungus *Penicillium* sp. F23-2 biosynthesized terpenoids, diketopiperazines, and meleagrin alkaloids when incubated in sea-water-based culture medium under static condition (Du et al., 2009, 2010), whereas five new nitrogen-containing sorbicillinoids (**238**–**242**) were metabolized by this stain when cultivated in PYG (peptone yeast glucose) medium under shaking status (Guo et al., 2013).

Pestalotiopsis

When grown in rice medium, one endophytic strain of *Pestalotiopsis fici* from *Camellia sinensis* was found to be a prolific producer of bioactive secondary metabolites, including pupukeanane chloride (**243**) (Liu et al., 2008a), chloropestolide A (**244**) (Liu et al., 2009a), seven isoprenylated chromones (**245**–**251**) (Liu et al., 2010), three highly functionalized compounds (**252**–**254**) (Liu et al., 2009b), and three cytotoxic pupukeanane chlorides (**255**–**257**) (Liu et al., 2011). *In vitro* cytotoxic assays suggested that compound **244** possessed potent inhibitory effects on HeLa and HT29 with GI_{50} values of 0.7, 4.2 μM, respectively. However, this strain produced new cyclopropane derivatives (**258**–**262**) when cultivated in shaking liquid medium (Liu et al., 2008b). An endophytic fungus *P. foedan*, residing in *Bruguiera sexangul*, synthesized a new reduced spiro azaphilone derivative (**263**) together with two new isobenzofuranones (**264**–**265**) in solid GYM (glucose, yeast extract, malt) medium (Ding et al., 2008). But, in liquid modified PDB medium, a pair of novel spiro-γ-lactone enantiomers (**266**–**267**) were identified (Yang and Li, 2013).

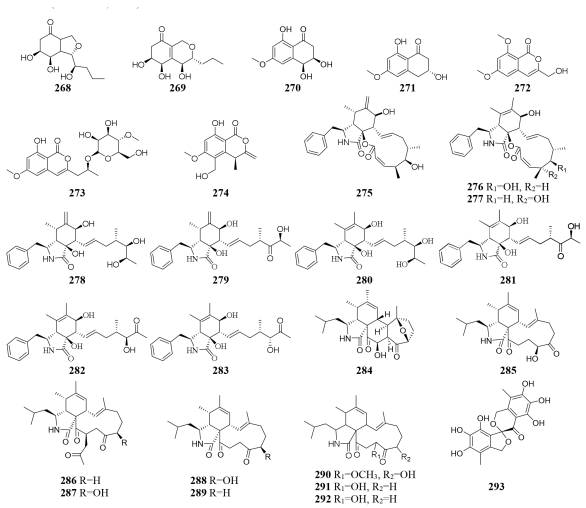


Phomopsis

An endophytic fungus *Phomopsis* sp. sh917 isolated from fresh stems of *Isodon eriocalyx var. laxiflora* collected in Kunming Botanical Garden of China, was shown to produce six new polyketides (**268**–**273**) on solid rice medium but metabolize a new polyketide (**274**) in shaking liquid FM4 medium (Tang et al., 2017).

Spicaria

Nine new cytochalasins Z7-Z15 (**275**–**283**), one novel spicochalasin (**284**), five new aspochalasins (**285**–**289**), and three new aspochalasin derivatives (**290**–**292**) were synthesized by a marine-derived fungus *Spicaria elegans* KLA03 in the seawater-based medium under static fermentation status. Compounds **235** and **276** displayed strong cytotoxicity against P388 and A-549 cancer cell lines with IC_{50} values in range of 8.4–99 μM (Liu et al., 2005, 2006, 2008c; Lin et al., 2009a, 2010). However, new aromatic polyketide (**293**) was obtained from shaking seawater medium (Luan et al., 2014).

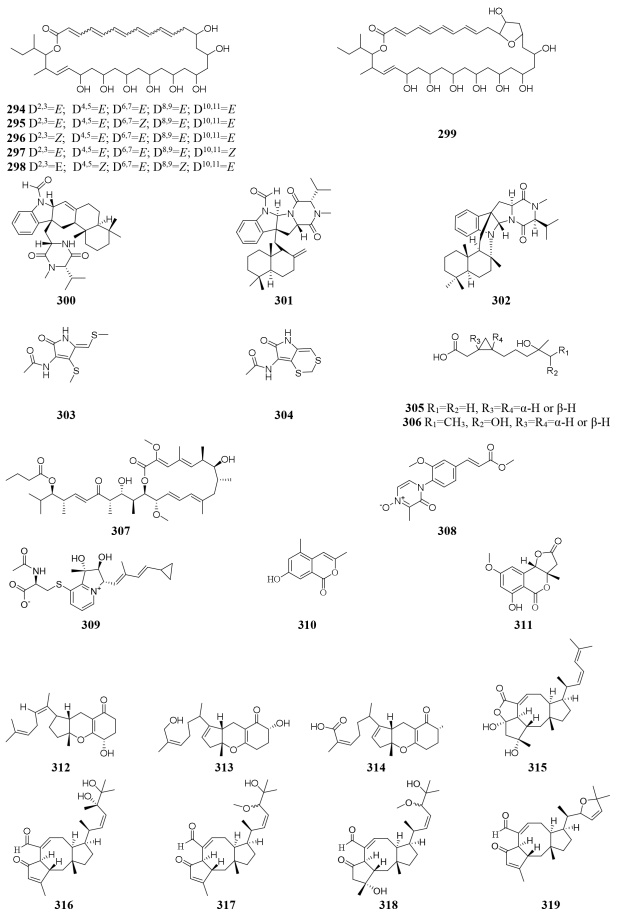


Streptomyces

One marine-derived stain *Streptomyces* sp. CHQ-64 was found to produce six new antifungal polyene-polyols (**294**–**299**) and two new cytotoxic hybrid isoprenoid alkaloids (**300**–**301**) in liquid medium under shaking condition, while this strain made only one new hybrid isoprenoid alkaloid (**302**) under static condition (Che et al., 2012, 2013, 2015, 2016). When cultivated in liquid Gause's No. 1 medium, strain *Streptomyces* sp. DT-A37 could produce a new ring-opened lactam (**303**), while in rice medium one unknown holomycin (**304**) and two new cyclopropaneacetic acids (**305**–**306**) were detected (Ding et al., 2017). Strain *Streptomyces* sp. HZP-2216E cultured in 2216E solid medium, GYM solid medium and GMSS (Gause's medium with sea salt) liquid medium resulted in isolation of two new compounds of 23-*O*-butyrylbafilomycin D (**307**), streptoarylpyrazinone A (**308**) a unique indolizinium alkaloid streptopertusacin A (**309**). It was noted that compound **307** showed potent activity in suppressing the proliferation of the four tested glioma cell lines with IC_{50} values in a range from 0.35 to 2.95 μM and antibacterial activity with MIC value of 7.4 μM for MRSA and IC_{50} values of 0.44 to 0.98 μM for glioma cells (Zhang et al., 2017c,d).

Ulocladium

Two antifungal polyketides (**310**–**311**) were characterized from rice medium of *Ulocladium* sp. that was isolated from the lichen *Everniastrum* sp. (Wang X.E. et al., 2012), whereas three new tricycloalternarenes F-H (**312**–**314**) and five ophiobolane sesterterpenes (**315**–**319**) were detected in liquid Czapek or PDB medium (Wang et al., 2013a,b). Compounds **315** and **319** exhibited moderate antibacterial activity against meticillin-resistant *S. aureus* and *Bacillus subtilis* and displayed strong *in vitro* cytotoxicity against cancer cell lines KB and HepG2.

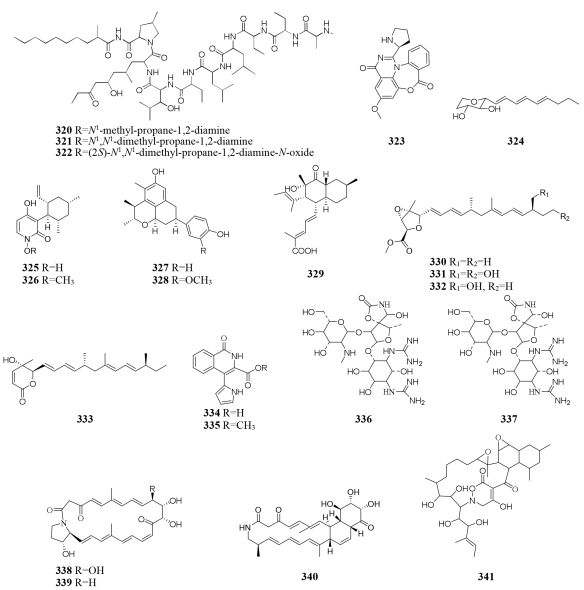


CO-CULTIVATION WITH OTHER STRAIN(S)

In one culture medium, the relationship between one strain and other(s) may be competitive, antagonistic or friendly. Co-cultivation of two or more strains usually has positive effect of an enhanced production of known compounds or an accumulation of cryptic compounds that are not detected in axenic culture (Bohni et al., 2013; Marmann et al., 2014). This effect maybe results from the production of enzymes that activate metabolite precursors or that other strain(s) may induce epigenetic modifications of the producer strain.

Fungus and Other Fungal Strain

An endophytic strain *Acremonium* sp. Tbp-5 from the European yew (*Taxus baccata* L.) could produce new lipoaminopeptides (**320**–**322**) when co-cultivated with *Mycogone rosea* DSM 12973 (Degenkolb et al., 2002). Chemical investigation of the mixed fermentation broth of two epiphytic strains *Aspergillus* sp. FSY-01 and FSW-02 from marine mangrove *Avicennia marina* led to the isolation of a novel alkaloid (**323**), which had antibacterial activity against *Bacillus dysenteriae*, *B. proteus*, and *E. coli* (Zhu et al., 2011). The production of 2-alkenyl-tetrahydropyran analogs (**224**–**326**) was provoked by *Chaunopycnis* sp. CMB-MF028 in the mixed culture with a partner strain *Trichoderma hamatum* CMB-MF030, which were isolated from the inner tissue of marine pulmonate false limpet (Shang et al., 2017). Co-cultivation of *Monascus* sp. J101, used as the producer of *Monascus* pigment, with *Saccharomyces cerevisiae* KCCM 11371 or *A. oryzae* KCCM 11773 on the solid sucrose medium could result in two folds of accelerated cell growth and 30–40 folds of increased pigment production (Shin et al., 1998). Strain J101 was shown to stimulate cell growth and reproduction by interacting with *S. cerevisiae*, which resulted in production of more hydrophobic pigments compared to the mono-culture (Suh and Shin, 2000a,b). When co-cultivated with *Beauveria felina*, one marine-derived *P. citrinum* could biosynthesize two new compounds (**327**–**328**) featuring in a unique tetracyclic framework, whereas neither strain could produce these compounds in axenic medium. Antimicrobial assay showed that compounds **327** and **328** had strong inhibitory effects on human pathogens *S. aureus* and *E. coli* (Meng et al., 2015a). *Penicillium* sp. IO1 derived from mediterranean sponge *Ircinia oros* could produce a new fusarielin analog (**329**). However, co-cultivation of *Penicillium* strains IO1 and IO2 resulted in the accumulation of two known compounds norlichexanthone and monocerin, which were not detected in axenic controls (Chen et al., 2015a). Four new polyketides (**330**–**333**) were detected in a dual culture of the deep-sea-derived fungus *Talaromyces aculeatus* and a mangrove-derived fungus



P. variabile, while these compounds were not identified in single culture. Compounds **333** displayed strong cytotoxicity against A549, K562, HCT-116, HeLa, MCF-7 and HL-60 human cancer cell lines with IC_{50} values ranging from 1.2 to 9.8 μM (Zhang et al., 2017b).

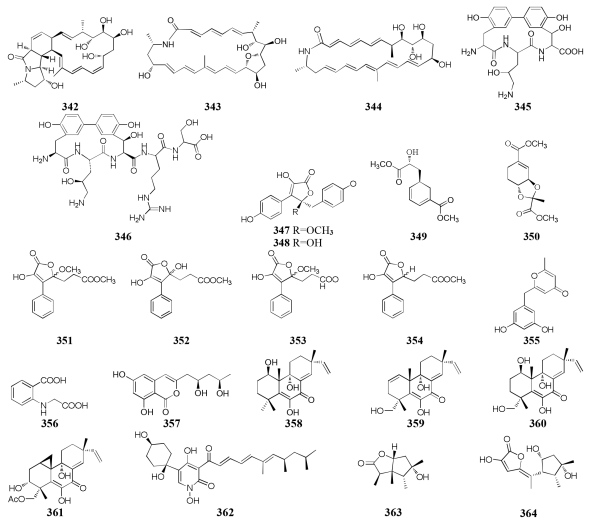
One novel 1-isoquinolone analog (**334**) and its methyl ester (**335**) were detected in mycelia and culture filtrate of mixed fermentation of two endophytic fungi Nos. 1924# and 3893#, whereas these compounds were not traced in axenic medium under the same conditions (Zhu and Lin, 2006). The formation of new antibiotics (**336**–**337**) was emerged during co-cultivation of a multi-antibiotic stable mutant strain of *Rhodococcus fascians* and a strain *Streptomyces padanus*, neither of which was capable of yielding an antibiotic (Kurosawa et al., 2008). An terrestrial bacterium *Tsukamurella pulmonis* TP-B0596 co-cultured with strain *Streptomyces* sp. NZ-6, coincided with stimulation of three new metabolites (**338**–**340**) of unprecedented di-andtricyclic macrolactams (Hoshino et al., 2015). The yield of red pigment was detected in the dual induction of *T. pulmonis* TP-B0596 and *S. lividans* TK23. Co-cultivation of *T. pulmonis* and *S. endus* S-522 resulted in the production of one new antibiotics called alchivemycin A (**341**) (Onaka et al., 2011). A soil-dwelling actinomycetes *S. coelicolor* was shown to significantly improve the yield of red compound undecylprodigiosin, when co-cultured with *Corallococcus coralloides* (Schäberle et al., 2014).

Bacterium and Other Bacterial Strain

Only two new macrolactams (**342**–**343**) were detected in the co-culture broth of a rare actinomycete *Micromonospora wenchangensis* HEK-797 and *Tsukamurella pulmonis* TPB0596, whereas the axenic medium of strain HEK-797 produced a polyene macrolactam (**344**), which was possibly the precursor of compounds **342** and **343** (Hoshino et al., 2017). Investigation of the interaction of the portable predator *Myxococcus Xanthus* and *Streptomyces coelicolor* showed that actinorhodin production of *S. coelicolor* was raised up to 20-fold and stimulated aerial mycelium production (Pérez et al., 2011). Co-cultivation of two sponge-derived actinomycetes, *Nocardiopsis* sp. RV163 and *Actinokineospora* sp. EG49, induced ten reported compounds, including diketopiperazine, angucycline, and β-carboline derivatives, while only three natural products were isolated in mono-culture (Dashti et al., 2014). Mixed culture of *Pseudomonas maltophilia* 1928 and *S. griseorubiginosus* 43708 resulted in the production of one peptide antibiotic, biphenomycin A (**345**) (Ezaki et al., 1992). However, the accumulation of biphenomycin A, which could be obtained from the transformation of biphenomycin C (**346**), was inhibited in single culture of strain 1928 (Uchida et al., 1985; Ezaki et al., 1993). Interspecies interactions between *Streptomyces coelicolor* M145 with other actinomycete stains (*Amycolatopsis* sp. AA4, *Streptomyces* sp. E14, *Streptomyces* sp., SPB74 and *S. viridochromogenes* DSM 40736) resulted in the production of at least 12 different versions of a molecule called desferrioxamine (Traxler et al., 2013).

Fungus and Bacterium

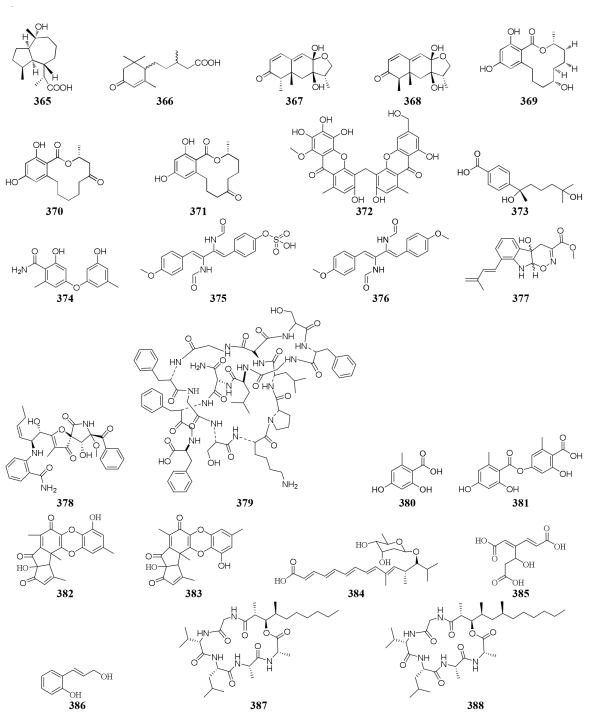
Co-cultivation of one fungal strain *A. terreus* with *B. cereus* and *B. subtilis* resulted in the yield of two novel butyrolactones (**347**–**348**), which were absent in single culture medium (Chen et al., 2015b). An endophyte *Chaetomium* sp. from the Cameroonian plant *Sapium Ellipticum* (Euphorbiaceae) was shown to produce two novel shikimic acid analogs (**349**–**350**) and four new butenolide derivatives (**351**–**354**) when co-cultivated with *Pseudomonas aeruginosa*, while none of these chemicals was traced in axenic medium (Ancheeva et al., 2017). Strain *Bacillus subtilis* 168 trpC2 was shown to greatly activate the biosynthesis of three novel chemicals (**355**–**357**) of fungal endophyte *Fusarium tricinctum* during co-culture process. And these compounds were not duplicated in axenic fungal culture (Ola et al., 2013).



Co-cultivation of one marine fungus *Libertella* sp. CNL-523 symbiotic on an ascidian collected from the Bahamas and a fellow strain *Thalassospira* sp. CNJ-328 resulted in the production of four new diterpenoids (**358**–**361**). Compound **360** exhibited remarkable cytotoxicity against HCT-116 human adenocarcinoma cell line with an IC_{50} value of 0.76 μM (Oh et al., 2005). A new pyridone alkaloid (**362**) was isolated from the mixed culture extract of *Paecilomyces lilacinus* and *Salmonella typhimurium*, which had 57.5 ± 5.50% of AChE inhibition (Teles and Takahashi, 2013). Co-culture of an endophyte *Pestalotiopsis* sp. from *Drepanocarpus lunatus* with *B. subtilis* was found to biosynthesize two novel sesquiterpenoids (**363**–**364**) while new compounds **365** and **366** emerged in axenic culture (Liu et al., 2017). The mixed cultivation of *Trichoderma* sp. 307 colonizing in *Clerodendrum inerme* and one bacterium *Acinetobacter johnsonii* B2 led to the production of two new sesquiterpenes (**367**–**368**) and three novel de-*O*-methyl lasiodiplodins (**369**–**371**). Compounds **369** and **370** displayed potent α-glucosidase inhibitory effect with IC_{50} values of 25.8 and 54.6 μM, respectively (Zhang et al., 2017a).

Chemical study of an endophytic stain *Aspergillus austroafricanus* from *Eichhornia crassipes* led to the isolation of a highly oxygenated heterodimeric xanthone (**372**) and a new sesquiterpene (**373**) in axenic culture. Mixed fermentation of *A. austroafricanus* with *B. subtilis* or *S. lividans* afforded several

diphenyl ethers, including one new austramide (**374**) (Ebrahim et al., 2016). Two novel *N*-formyl alkaloids (**375–376**) were characterized from a mixed fermentation of *A. fumigatus* and *S. peucetius*. Compound **376** displayed *in vitro* cytotoxic effect on cancer cell line NCI-60 with an IC_{50} value of 1.12 μM (Zuck et al., 2011). Seven known diketopiperazine alkaloids associated with ergosterol and 11-*O*-methylpseurotin A were traced in response to the supplement of *A. fumigatus* MBC-F1-10 to an established culture of *S. bullii*, whereas neither strain metabolized these compounds when cultivated alone (Rateb et al., 2013). Co-cultivation of *A. fumigatus* MR2012 with *S. leeuwenhoekii* C34 in ISP2 medium resulted in the yield of a new luteoride derivative (**377**) and a new pseurotin derivative (**378**). None of these compounds could be detected in axenic culture. When strain MR2012 was co-cultivated with strain C58, a lasso peptide chaxapeptin (**379**) was made, which displayed significant inhibitory effect on human lung cancer cell line A549 (Elsayed et al., 2015; Wakefield et al., 2017).



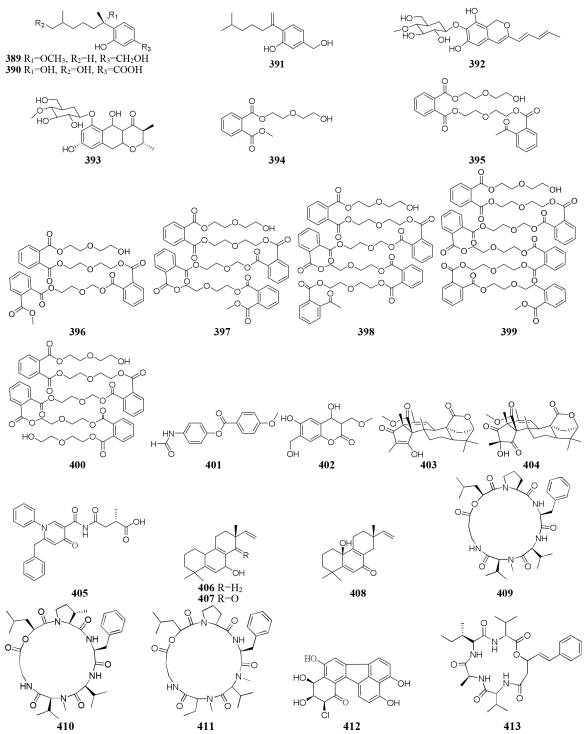
Physical interaction of *A. nidulans* RMS011 with *S. hygroscopicus* was found to trigger biosynthesis of four new aromatic polyketides (**380–383**), which were absent in the axenic medium (Schroeckh et al., 2009). A new polyketide glycoside (**384**) was formed in the dual induction of two Gram-positive bacteria, *S. tendae* KMC006 and *Gordonia* sp. KMC005, which were obtained from an acidic mine drainage sample (Park et al., 2017). In response to *S. coelicolor* A3(2) M145, strain *A. niger* N402 was shown to be apt to produce 2-hydroxyphenylacetic acid and cyclic dipeptide cyclo(Phe–Phe). Biotransformation of a new hexadienedioxic acid (**385**) and a new phenol derivative (**386**) was achieved by co-culture of these strains (Wu et al., 2015). More interestingly, co-cultivation of one marine-derived fungus *Emericella* sp. CNL-878 with *Salinispora arenicola* CNH-665 resulted in the higher yields of two novel antimicrobial cyclic depsipeptides (**387–388**) than axenic culture (Oh et al., 2007).

EPIGENETIC MODIFIER

Epigenetic modifiers are those chemicals that are able to change microbial characteristics in correspondence to alteration of their epigenetic status, such as DNA methyltransferase (DNMT) inhibitor and histone deacetylase (HDAC) inhibitor. The addition of these modifiers usually suppresses the activity of related enzymes in the biosynthetic pathway and promotes the progress of other metabolic pathways (Seyedsayamdost, 2014).

DNA Methyltransferase Inhibitor

DNA methylation is a process by which methyl groups are added to DNA. When located in a gene promoter, DNA methylation typically acts to repress gene transcription and causes chromatin structure changes in the corresponding regions, preventing the binding of specific transcription factors and suppressing gene expression (Araujo et al., 2001). 5-Azacytidine (5-AC) is the most common DNMT inhibitor used to modify the function of microbe DNA followed by repressing gene transcription. Chemical investigation of a marine-derived fungus *Aspergillus sydowii* afforded three novel bisabolane-type sesquiterpenoids (**389–391**) when its culture medium was supplemented with 5-AC (Chung et al., 2013b). An entomopathogenic fungus *Cordyceps indigotica* yielded a novel aromatic polyketide

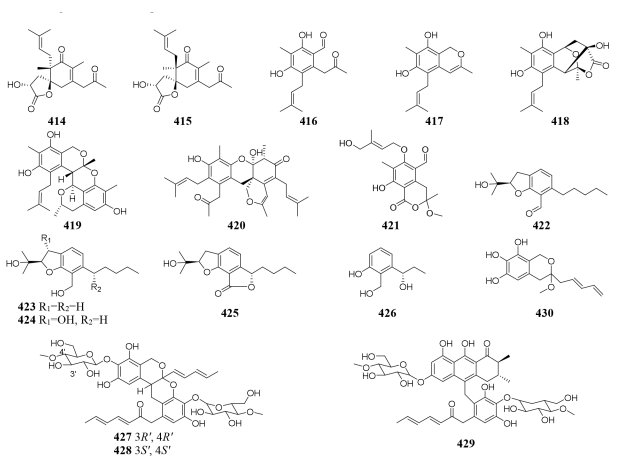


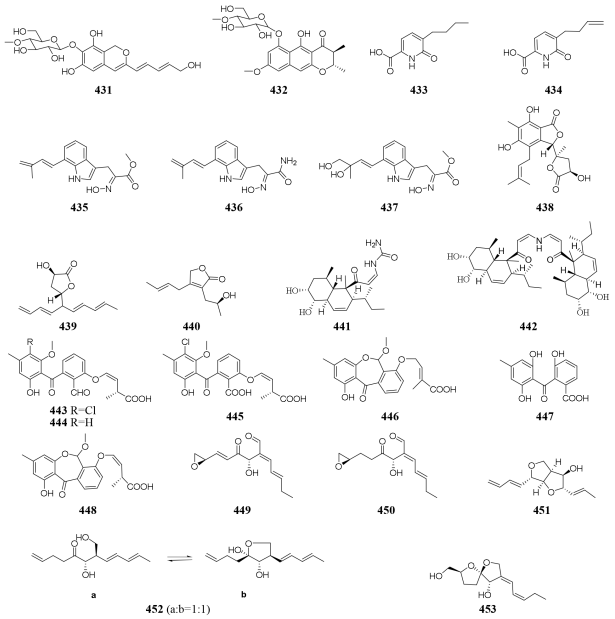
glycoside (**392**) when cultivated in PDB media, while the strain produced another unusual glycoside (**393**) when supplement with 5-AC (Asai et al., 2012e). Several other examples that adding 5-AC as epigenetic modifier in culture medium could lead to the production of new metabolites were also reported, such as novel diethylene glycol phthalate esters (**394**–**400**) from a marine-derived strain *Cochliobolus lunatus* TA26-46 (Chen et al., 2016), a new benzoic acid (**401**) from *Pestalotiopsis microspora* (Yang et al., 2017), one new coumarin (**402**) from *P. crassiuscula* NBRC 31055 associated with *Fragaria chiloensis* (Yang et al., 2014), and novel meroterpenes (**403**–**404**) from *P. citreonigrum* (Wang et al., 2010).

Histone Deacetylase Inhibitor

The acetylation or deacetylation of histone affects its binding to DNA in microbe. There are many chemical modifications in the tail of histone that regulate the gene expression. The introduction of hydrophobic acetyl group into the *N*-terminal lysine residues of histone could increase the electrostatic attraction and steric hindrance between histone and DNA, which is conducive to facilitate the depolymerization of DNA and the binding of transcription factors (Fukuda et al., 2006; Cole, 2008). Suberoyl bishydroxamic acid (SBHA), suberoylanilide hydroxamic acid (SAHA), and nicotinamide are the most common HDAC chemicals used to inhibit the deacetylation and facilitate gene transcription and expression in microbes (Moore et al., 2012).

Many reports suggested the presence of SAHA in culture medium could result in production of new natural compounds, such as a novel metabolite nygerone A (**405**) from a soil-dwelling fungus *A. niger* ATCC 1015 (Henrikson et al., 2009), two new aromatic norditerpenes (**406**–**407**) tied with an oxygenated derivative (**408**) from a marine-derived *A. wentii* na-3 residing in the brown alga *Sargassum fusiforme* (Miao et al., 2014), three novel cyclodepsipeptides (**409**–**411**) from *Beauveria feline* (Chung et al., 2013a), one novel chlorinated polyketide (**412**) from *Daldinia* sp. (Du et al., 2014), a new cyclodepsipeptide of hybrid EGM-556 (**413**) from one marine sediment-derived fungus *Microascus* sp. (Vervoort et al., 2011).





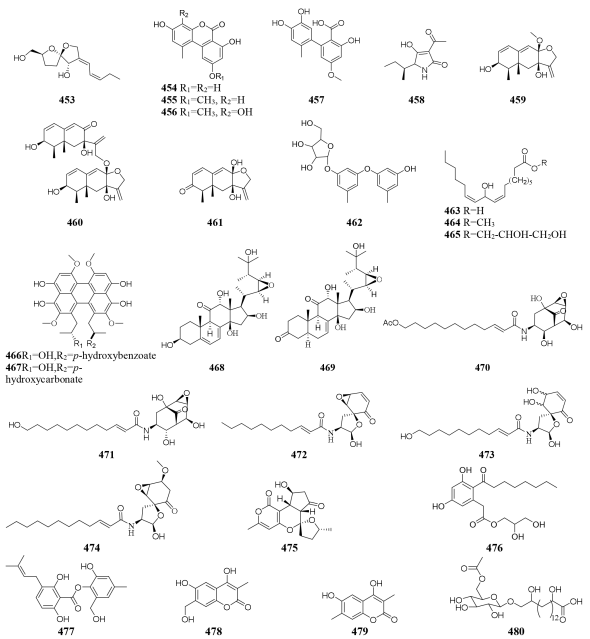
In SBHA-treated culture medium, *Chaetomium indicum* could produce two novel spironolactone polyketides (**414**–**415**) and six novel prenylated aromatic polyketides (**416**–**421**) (Asai et al., 2013b,c). Similarly, when exposed to SBHA, four new 2,3-dihydrobenzofurans (**422**–**425**) and a new aromatic polyketide (**426**) were characterized from an entomopathogenic fungus *Cordyceps annullata* (Asai et al., 2012c), six new aromatic polyketides (**427**–**432**) were synthesized by *C. indigotica* (Asai et al., 2012f), two new fusaric acid derivatives (**433**–**434**) were produced by *Fusarium oxysporum* associated with medicinal plant *Datura stramonium* L. (Chen et al., 2013), and a series of novel prenylated tryptophan analogs (**435**–**437**) were metabolized by an entomopathogenic fungus *Torrubiella luteorostrata* (Asai et al., 2011). Supplement of nicotinamide [a Zn(II)-type HDAC inhibitor] in culture medium of *C. cancroideum* could generate three novel polyketides (**438**–**440**) (Asai et al., 2016). The use of this inhibitor to strains *Eupenicillium* sp. LG41 and *Graphiopsis chlorocephala* had the similar effect, which the former supplied two new decalin-containing compounds (**441**–**442**) (Li G. et al., 2017) and the later afforded a serious of new benzophenones (**443**–**444**) and diverse new C^{13}-polyketides (**445**–**453**) (Asai et al., 2012d, 2013a).

Multiple Chemical Epigenetic Modifiers

Interactions between epigenetic features play an important role in regulation of gene expressing or silencing in microorganisms, such as DNA methylation and histone modification. Many references that looked into the combined effect of epigenetic processes suggested that these chemicals could regulate the activity of genomic regions of varying sizes, from single genes to entire domains and chromosomes. Epigenetic markers could also interact with other nuclear proteins to work together to form chromatin structures and to create genomic functional discrete

regions that induce the production of new secondary metabolites (Tammen et al., 2013).

One symbiotic strain *Alternaria* sp. from medicinal plant *Datura stramonium Linn.* was shown to produce four new aromatic polyketides (**454**–**457**) and a new tenuazonic acid (**458**) when incubated in medium containing 5-AC and/or SBHA. While these compounds were absent in normal culture medium. Interestingly, the yield of these secondary metabolites was higher in the medium of adding HDAC and DNMT inhibitors than that of addition of any other inhibitors (Sun et al., 2012). Chemical investigation of one marine-derived fungus *Aspergillus* sp. SCSIOW2 or SCSIOW3, exposed with an integration of SHBA and 5-AC, led to production of three new eremophilane-type sesquiterpenes (**459**–**461**) together with a new diphenylether-*O*-glycoside (**462**) (Wang L. et al., 2016; Li X. et al., 2017). Bioactivity tests indicated that the glycosylated compound **462** exhibited a protective activity toward free radicals with an EC_{50} value of 20.8 μM. One strain *Cladosporium cladosporioides* from a tidal pool was found to display different responses to the treatment with 5-AC and SHBA. Exposure of *C. cladosporioides* to 5-AC resulted in substantially increased biosynthesis of three oxylipins (**463**–**465**), whereas SHBA induced the yield of two new perylenequinones (**466**–**467**) (Williams et al., 2008).



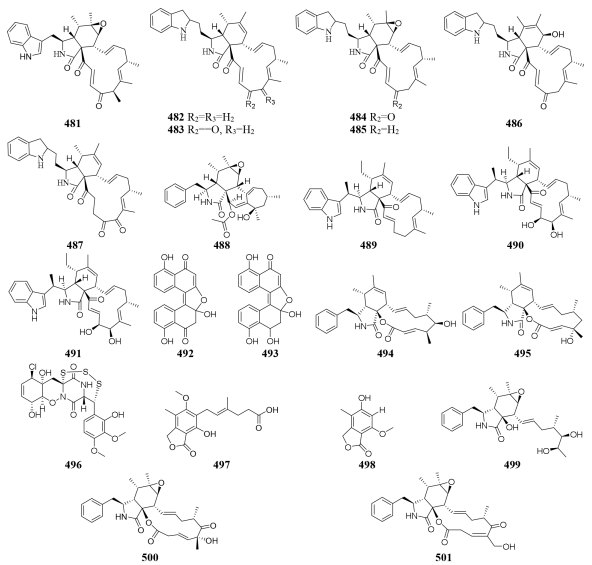
Concomitant supplement of SHBA and *N*-phthalyl-*L*-tryptophan (DNMT inhibitor) to the fermentation medium of an entomopathogenic fungus *Gibellula formosana* induced the formation of two new highly oxidized ergosterols (**468**–**469**) and five new isariotin analogs (**470**–**474**) (Asai et al., 2012a). The same method was applied to expand MSM profile of *Isaria tenuipes*, which resulted in the yield of one new polyketide (**475**) (Asai et al., 2012b). An endophytic strain *Leucostoma persoonii* from red mangrove was subject to large-scale cultivation with sodium butyrate (HDAC inhibitor) and 5-AC, which resulted in the increased yield of known cytosporones and the production of one new cytosporone (**476**) (Beau et al., 2012). Three novel aromatics (**477**–**479**) were produced by *Pestalotiopsis acacia* from *Taxus brevifolia* when its culture medium was supplemented with SHBA and 5-AC (Yang and Li, 2013). Application of this approach also led to production of a new glycolipid ustilagic acid C (**480**) by *Ustilago maydis* (Yang et al., 2013).

OTHER FACTORS

Enzyme Inhibitor

Beside DNMT and HDAC, other microbial enzymes also played important role in the regulating the biosynthesis of secondary metabolites, such as monooxygenase and hydroxylase. Some chemicals can selectively inhibit the activity of these enzymes in the biosynthetic pathway and promote the progress of other metabolic pathways, such as metyrapone, tricyclazole, jasplakinolide, and DMSO.

Chemical study of *Chaetomium subaffine* in the presence of metyrapone (an inhibitor of cytochrome P-450) led to purification of five new polyketides (**481**–**485**) and two new less oxidized analogs (**486**–**487**) (Oikawa et al., 1992). A soil-derived strain *Phoma* sp. SNF-1778 was shown to yield a new cytochalasin (**488**) when inoculated with metyrapone (Kakeya et al., 1997). When added with the F-actin inhibitor jasplakinolide in culture medium, one marine sponge-derived fungus *Phomopsis asparagi* could afford three unusual cytotoxic compounds, chaetoglobosin-510 (**489**), chaetoglobosin-540 (**490**), and chaetoglobosin-542 (**491**) (Christian et al., 2005). Two novel bisnaphthalene compounds (**492**–**493**) were characterized from *Sphaeropsidales* sp. F-24'707 cultured with tricyclazole, which was shown to inhibited the regular biosynthesis of 1,8-dihydroxynaphthalene (Bode and Zeeck, 2000). Continuous study showed that metyrapone supplementation in the culture of *Spicaria elegans* led to the isolation of two novel 7-deoxy-cytochalasins (**494**–**495**). Compound **494** had weak



cytotoxicity against human lung cancer cell line A-549 at 15.0 mM (Lin et al., 2009b). One marine-derived strain *Trichoderma* cf. *brevicompactum* elicited an unprecedented epidiketopiperazine (**496**), which has a trisulfide bond between the α–β positions of two amino acid residues, by adding DMSO to its natural seawater medium (Yamazaki et al., 2015b).

Biosynthetic Precursor

Biosynthetic precursor refers to one chemical that is apt to be directly incorporated into the final product. Adding various biosynthetic precursors in the fermentation medium may change biosynthesis pathways of secondary metabolites and result in the production of novel compounds (Ramm et al., 2017).

An endophytic strain *Penicillium crustosum* from the ripe berry of *Coffea arabica* L., treated with ferulic acid and quinic acid or cinnamic acid and 3,4-(methylenedioxy) cinnamic acid, was shown to produce mycophenolic acid (**497**) and 5-hydroxy-7-methoxy-4-methylphthalide (**498**) (Valente et al., 2013). Three novel cytochalasins Z_{21}–Z_{23} (**499**–**501**) were characterized from one marine-derived fungus *Chaetomium indicum* KLA03 when cultivated in medium supplied by L- and D-tryptophan. Compound **498** exhibited potent cytotoxic effect on A549 cell lines with an IC_{50} value of 8.2 μM (Wang F. Z. et al., 2011). Strain *S. griseoviridis* Tü 3634 could afford a wide variety of acyl α-L-rhamnopyranosides (pyrrolyl, indolyl, thienyl, furanyl, and pyridyl derivatives) if its culture medium, respectively, added corresponding precursors, heteroaromatic carboxylic acid, benzoic acid, cinnamic acid, aminobenzoic acid, and salicylic acid (Grond et al., 2000, 2002).

CONCLUSION

Microorganisms are susceptible to culture conditions, such as medium composition, temperature, pH, salinity, culture status, axenic or mixed culture, epigenetic modifier, biosynthetic precursor, and so on. Variation of these factors may result in changing chemical diversity of secondary metabolites. Traditional culture method of microbe is limited to the expression of a large number of metabolic pathways that many MSMs could not be biologically synthesized. A growing body of evidence has suggested that OSMAC strategy can provide a simple, quick and effective approach for enhancing chemodiversity of MSM to obtain new drug leads through activating silent gene clusters. Moreover, employment of this strategy could avoids the waste of time and resources caused by multiple inoculation, screening, culturing and separation in comparison with mutation strategy (Fang et al., 2014) and ribosome engineering (Ochi et al., 2004). Nowadays, the rate of discovery of new MSM is getting lower and the possibility of the rediscovery of known compounds is higher than before. Therefore, OSMAC strategy would be an important alternative way to alleviate this challenge. There is a great need for new method to assist in isolating and identifying novel bioactive MSMs, such as bioassay-guided isolation, microbe genomes mining (Hug et al., 2018) and LC-MS/MS based molecular networking analysis (Wang M. et al., 2016).

AUTHOR CONTRIBUTIONS

RP made a draft of the review. XB and JC searched and collected the references. HZ and HW conceived and revised this review.

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REVIEW
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A Review of the Microbial Production of Bioactive Natural Products and Biologics

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A variety of organisms, such as bacteria, fungi, and plants, produce secondary metabolites, also known as natural products. Natural products have been a prolific source and an inspiration for numerous medical agents with widely divergent chemical structures and biological activities, including antimicrobial, immunosuppressive, anticancer, and anti-inflammatory activities, many of which have been developed as treatments and have potential therapeutic applications for human diseases. Aside from natural products, the recent development of recombinant DNA technology has sparked the development of a wide array of biopharmaceutical products, such as recombinant proteins, offering significant advances in treating a broad spectrum of medical illnesses and conditions. Herein, we will introduce the structures and diverse biological activities of natural products and recombinant proteins that have been exploited as valuable molecules in medicine, agriculture and insect control. In addition, we will explore past and ongoing efforts along with achievements in the development of robust and promising microorganisms as cell factories to produce biologically active molecules. Furthermore, we will review multi-disciplinary and comprehensive engineering approaches directed at improving yields of microbial production of natural products and proteins and generating novel molecules. Throughout this article, we will suggest ways in which microbial-derived biologically active molecular entities and their analogs could continue to inspire the development of new therapeutic agents in academia and industry.

Keywords: natural products, biologics, biological activity, microbial cell factories, genetic engineering, combinatorial biosynthesis, production improvement

INTRODUCTION

Natural products originate as secondary metabolites from a myriad of sources, including terrestrial plants, animals, marine organisms, microorganisms, terrestrial vertebrates and invertebrates (Chin et al., 2006). These structurally and chemically diverse molecules act as a remarkable class of therapeutics to heal various ailments. The earliest documentation of the application of natural

products to improve human health dates back to the ancient Mesopotamia's sophisticated medicinal system from 2900 to 2600 BCE (Borchardt, 2002; Siddiqui et al., 2014). By the early 1900's, approximately 80% of all medicines were obtained from plant sources (Sneader, 1997; Siddiqui et al., 2014). The discovery of penicillin from *Penicillium notatum* by Alexander Fleming in 1928 marked a significant shift from plants to microorganisms as a source of natural products (Fleming, 1944). Since then, microorganism-derived compounds have been utilized in medicine, agriculture, food industry and scientific research (Sanchez et al., 2012). The early years of antibiotic research discovered streptomycin from *Streptomyces griseus* (Waksman et al., 1946), chloramphenicol from *Streptomyces venezuelae* (Duggar, 1948), chlortetracycline from *Streptomyces aureofaciens* (Ehrlich et al., 1947), cephalosporin C from *Cephalosporium acremonium* (Newton and Abraham, 1955), erythromycin from *Saccharopolyspora erythraea* and vancomycin from *Amycolatopsis orientalis* (Geraci et al., 1956). Given these historical successes, large pharmaceutical companies have continued to invest in this traditional domain (Dias et al., 2012). Currently, approximately 60% of approved small molecule medicines are related to natural products, and 69% of all antibacterial agents originate from natural products (Patridge et al., 2016; Matsumura et al., 2018). However, many natural compounds with potential as novel drug candidates occur in low concentrations in nature, often making drug discovery and development burdensome and economically impractical. Therefore, an emerging alternative solution is to express biosynthetic genes from the original producers in microbial hosts, notably bacteria and fungi (Song et al., 2014). Engineered microbes can produce appreciable amounts of scarce natural compounds, thereby facilitating the synthesis of the target novel compound and potent derivatives, as well as the validation of their activities (Matsumura et al., 2018).

The natural product sector is not the only area that has undergone substantial growth or utilizes therapeutic products generated in/from living organisms. Prokaryotic and eukaryotic microbial cells, in combination with the advancement of recombinant DNA techniques, have been responsible for an explosion of biologics. Biologics are a set of molecules whose active pharmaceutical ingredients are derived from living organisms such as animals, plants, microorganisms, human blood products, and tissue transplants that are too complex to be produced through organic synthesis (Revers and Furczon, 2010). They can be categorized into five main classes: (1) monoclonal antibodies, like trastuzumab (Herceptin®) and rituximab (Rituxan®); (2) blood factor derivatives, like coagulation factor VIIa (NovoSeven RT®) and epoiten alfa (Epogen®); (3) vaccines; (4) enzymes; and (5) recombinant proteins, such as immunomodulatory cytokines, and thrombolytic agents (Lacana et al., 2007). Since the approval of recombinant human insulin and recombinant human growth hormone as some of the first modern biopharmaceuticals, large numbers of additional biopharmaceuticals have been developed, approved, and marketed using different microbial expression systems; many more are currently in the development pipeline (Graumann and Premstaller, 2006). After the successful production of the recombinant human insulin Humulin®, *Escherichia coli* quickly became the prevalent expression platform in the 1980s when the biopharmaceutical sector emerged and was followed by yeast *Saccharomyces cerevisiae* (Sanchez-Garcia et al., 2016). Microbial cells constitute the majority of hosts employed in the production of currently approved recombinant pharmaceuticals for human treatment, mainly because of their lack of unconventional post-translational modifications, proteolytic instability, poor solubility and activation of cell stress responses (Graumann and Premstaller, 2006). This demonstrates that microbial hosts represent convenient and robust platforms for the efficient production of recombinant proteins despite some bottlenecks and obstacles.

Herein, we will summarize the biological activities and applications of a variety of natural products and biologics and review the microbial systems used to produce these pharmaceutical compounds. We will also cover past and current attempts at improving the microbial production of these biological molecules and generating new molecules using diverse engineering approaches. In addition, we will discuss the challenges of the production of natural products and biologics in microbial systems and advances that can help overcome them for drug discovery and development. Future prospects for cutting-edge developments and technological advances in microbial production of bioactive natural products and recombinant proteins as the most valuable sources of therapeutics are also discussed.

BIOLOGICAL ACTIVITIES OF NATURAL PRODUCTS AND BIOLOGICS

Natural products have diverse biological activities relevant to human health, including antibiotic, antifungal, anticancer, immunosuppressive, anti-inflammatory, biofilm inhibitory activities, etc. In this section, we will focus on the biological activities of natural products, which can be grouped into several categories. The biological activities of microbial recombinant proteins will be also reviewed.

Antibiotics

Natural products are a rich source for antibiotic drug development, but the most clinically useful of these scaffolds can be classified as polyketides, non-ribosomal peptides, and aminoglycosides (Wright, 2014). Polyketides, assembled by polyketide synthases (PKS), make up one of the largest classes of chemically diverse natural products and are among the most important secondary metabolites for their applications in medicine, agriculture, and industry (Tae et al., 2007). For example, pikromycin was the first known polyketide antibiotic produced from *S. venezuelae* in 1950 (Vazquez, 1967; Jung et al., 2006). It has been reported that pikromycin is very potent against multi-drug resistant respiratory pathogens (Woo et al., 2014). Another remarkable polyketide antibiotic with significant clinical applications is erythromycin A (1; **Figure 1** and **Table 1**), which was first discovered in 1952 as a broad-spectrum antibiotic produced by *S. erythraea* (McGuire et al., 1952). This antibiotic

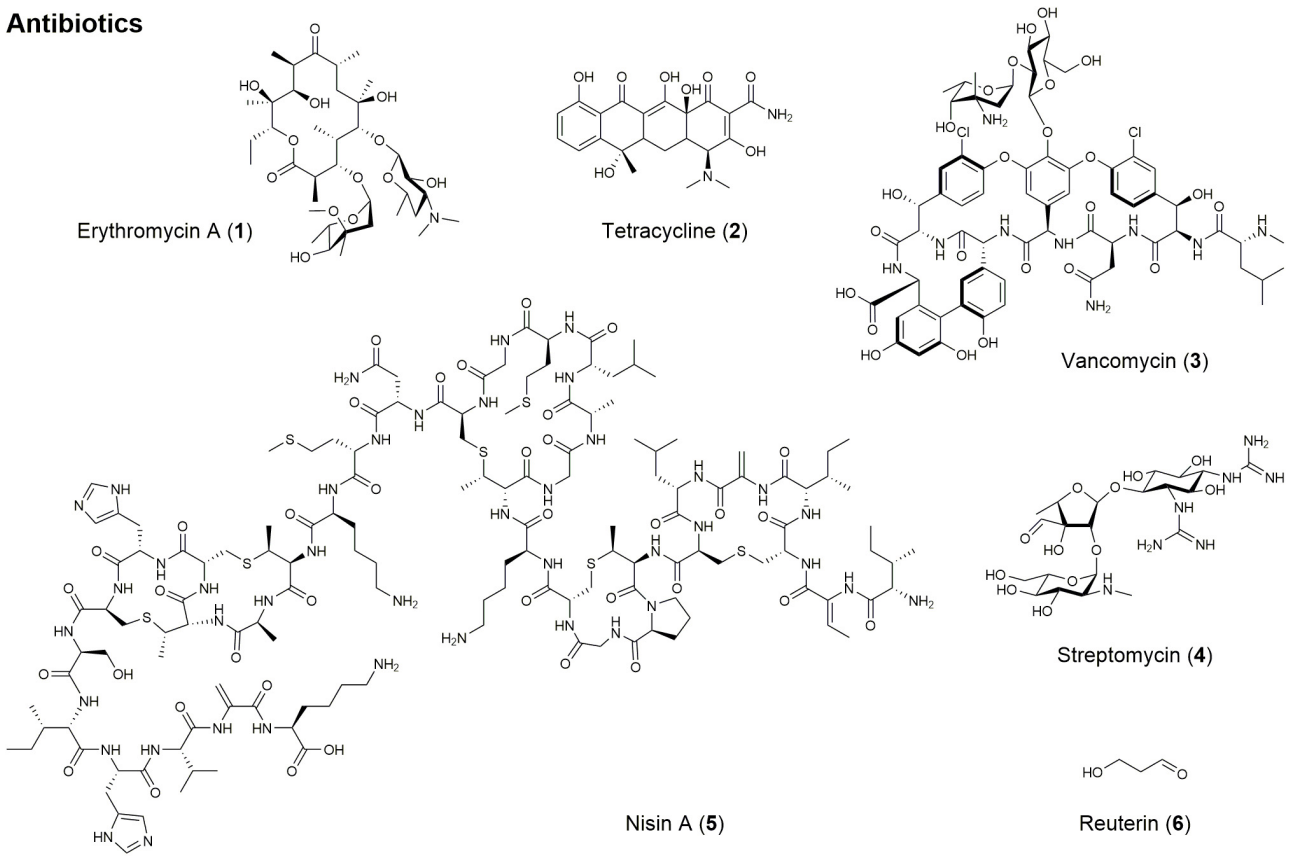

FIGURE 1 | Structures of natural products with antibiotic activity.

is prescribed to a treat wide variety of bacterial infections, such as respiratory and gastrointestinal infections, whooping cough, syphilis, and acne, especially in patients who have adverse reactions against penicillin (Cobb et al., 2013). While many natural antibiotics fail to inhibit Gram-negative organisms, tetracyclines (2; **Figure 1** and **Table 1**) are active against both Gram-positive and Gram-negative bacteria (Chopra and Roberts, 2001; Demain, 2009).

As previously mentioned, penicillin is a well-known antibiotic secondary metabolite from *P. notatum* and is effective against Gram-positive bacteria, which are responsible for diseases such as scarlet fever, pneumonia, gonorrhea, meningitis, and diphtheria (Fleming, 2001; Tan and Tatsumura, 2015). Penicillin belongs to non-ribosomal peptide antibiotics along with vancomycin (Fischbach and Walsh, 2006). Non-ribosomal peptides, assembled by non-ribosomal peptide synthetase (NRPS), possess bioactivity that can be exploited for therapeutic applications and are amongst the most widespread and structurally diverse secondary metabolites. Vancomycin (3; **Figure 1** and **Table 1**) is another potent non-ribosomal peptide against pathogenic bacteria, including *Clostridium difficile*, *Listeria monocytogenes*, *Streptococcus pneumoniae*, *Staphylococcus epidermidis*, and methicillin-resistant *Staphylococcus aureus* (MRSA) (Dasgupta, 2012).

Aminoglycosides are another class of antibiotics that act by binding to the rRNA subunit of the 30S bacterial ribosome and inhibiting protein synthesis (Moazed and Noller, 1987). Streptomycin (4; **Figure 1** and **Table 1**) produced by *S. griseus* is the first aminoglycoside discovered in 1944 and effective against pulmonary tuberculosis (Schatz et al., 1944). Since the discovery of streptomycin, aminoglycoside antibiotics such as kanamycin, gentamicin, sisomicin, and lividomycin have been discovered and widely used to treat infectious organisms that have developed resistance against streptomycin after prolonged use (Park et al., 2013). Despite their excellent antibacterial activity, aminoglycosides have met with resistant organisms. In order to combat antibiotic resistance to aminoglycoside antibiotics, semi-synthetic aminoglycoside antibiotics were specifically tailored to shield against these enzymes (Van Bambeke et al., 2017). Semi-synthetic aminoglycoside antibiotics such as amikacin, netilmicin, dibekacin, and isepamicin are developed as a result of semi-synthetic derivatives of the natural product (Miller et al., 1976; Leggett, 2015).

Natural antimicrobials have also been important to the food industry in terms of food safety against foodborne pathogens. Microbes such as lactic acid bacteria, produce a wide range of chemicals that have been shown to inhibit the growth and development of other microbial species. Nisin A (5; **Figure 1**

TABLE 1 | Biological activities of microbial-derived natural products and biologics.

Name	Origin	Biological activity	References
Antibiotic			
Erythromycin A (1)	*Saccharopolyspora erythraea*	Antibacterial	McGuire et al., 1952; Zhang et al., 2010; Cobb et al., 2013
Tetracycline (2)	*Streptomyces rimosus*	Antibacterial	Chopra and Roberts, 2001; Demain, 2009
Vancomycin (3)	*Amycolatopsis orientalis*	Antibacterial	Geraci et al., 1956; Dasgupta, 2012
Streptomycin (4)	*Streptomyces griseus*	Antibacterial	Schatz et al., 1944; Waksman et al., 1946
Nisin A (5)	*Lactococcus lactis*	Antimicrobial	Li and Vederas, 2009; Gyawali and Ibrahim, 2014
Reuterin (6)	*Lactobacillus reuteri*	Antimicrobial	Talarico et al., 1988; Gyawali and Ibrahim, 2014
Antifungal Agents			
Amphotericin B (7)	*Streptomyces nodosus*	Antifungal	Abu-Salah, 1996; Tevyashova et al., 2013
Ieodoglucomide C (8)	*Bacillus licheniformis*	Antifungal	Tareq et al., 2015
Anticancer and Antitumor			
Bleomycin (9)	*Streptoalloteichus hindustanus*, *Streptomyces verticillus*	Squamous cell carcinomas, Hodgkin's lymphomas and testis tumors	Einhorn and Donohue, 2002; Demain and Vaishnav, 2011
Ddaunorubicin (10)	*Streptomyces peucetius* and various related strains	Acute lymphoblastic or myeloblastic lymphoma	Di Marco et al., 1981; Giddings and Newman, 2013
Immunosuppressant/Anti-inflammatory Agents			
Rapamycin (11)	*Streptomyces rapamycinicus* (formerly, *Streptomyces hygroscopicus* ATCC 29253), *Streptomyces iranensis*, and *Actinoplanes* sp. N902-109	Immunosuppressive, antifungal, antitumor, neuroprotective, neuroregenerative, and lifespan extension activities, growth inhibitory activity against several fungi	Vezina et al., 1975; Mann, 2001; Law, 2005; Pan et al., 2008; Anisimov et al., 2011; Song et al., 2015; Yoo et al., 2017
FK506 (12)	*Streptomyces tsukubaensis* and several *Streptomyces* species	Immunosuppressive, antifungal, anti-inflammatory, neuroprotective and neuroregenerative activities, rheumatoid arthritis treatment	Tanaka et al., 1987; Mann, 2001; Migita and Eguchi, 2003; Demain, 2014; Ban et al., 2016; Yoo et al., 2017
Biofilm-Inhibitory Agents			
Cahuitamycins (13)	*Streptomyces gandocaensis*	Inhibitors of *Acinetobacter baumannii* biofilms	Park et al., 2016
Others			
Avermectins (14)	*Streptomyces avermitilis*	Onchocerciasis and lymphatic filariasis	Shen, 2015
Mollemycin A 20 (15)	*Streptomyces* sp. (CMB-M0244)	Gram-positive and Gram-negative bacteria, antimalarial activity	Blunt et al., 2016
Lipstatin (16)	*Streptomyces toxytricini*	Pancreatic lipase inhibitor for obesity and diabetes	Weibel et al., 1987; Sanchez et al., 2012

and **Table 1**), a bacteriocin produced by *Lactococcus lactis*, is approved to preserve food in over 50 countries and is very active against Gram-positive bacteria resistant to conventional antibiotics (Li and Vederas, 2009; Gyawali and Ibrahim, 2014). Reuterin (6; **Figure 1** and **Table 1**) from *Lactobacillus reuteri* has been shown to have antimicrobial activities against foodborne pathogens and spoilage organisms when evaluated in milk, dairy, and meat products (Talarico et al., 1988; Gyawali and Ibrahim, 2014).

Antifungal Agents

Nystatin, one of the first effective polyene antifungal agent, was obtained from *Streptomyces noursei* in 1950 and effective against *Aspergillus* species (Stanley and English, 1965). Clinically, nystatin plays a significant role as a topical antifungal agent in treating oral, gastro-intestinal, and genital candidosis (Fjærvik and Zotchev, 2005). Amphotericin B (7; **Figure 2** and **Table 1**) is also a traditional polyene antifungal product of *Streptomyces nodosus* utilized against life-threatening fungal infections caused by *Aspergillus* species, and especially effective in patients who have undergone organ transplantation, received aggressive chemotherapy or with acquired immunodeficiency syndrome (Abu-Salah, 1996; Tevyashova et al., 2013).

Recently, in a review of natural products with anti-*Candida albicans* activity, 71 substances of the 142 evaluated were determined to have antifungal activity under the criteria of

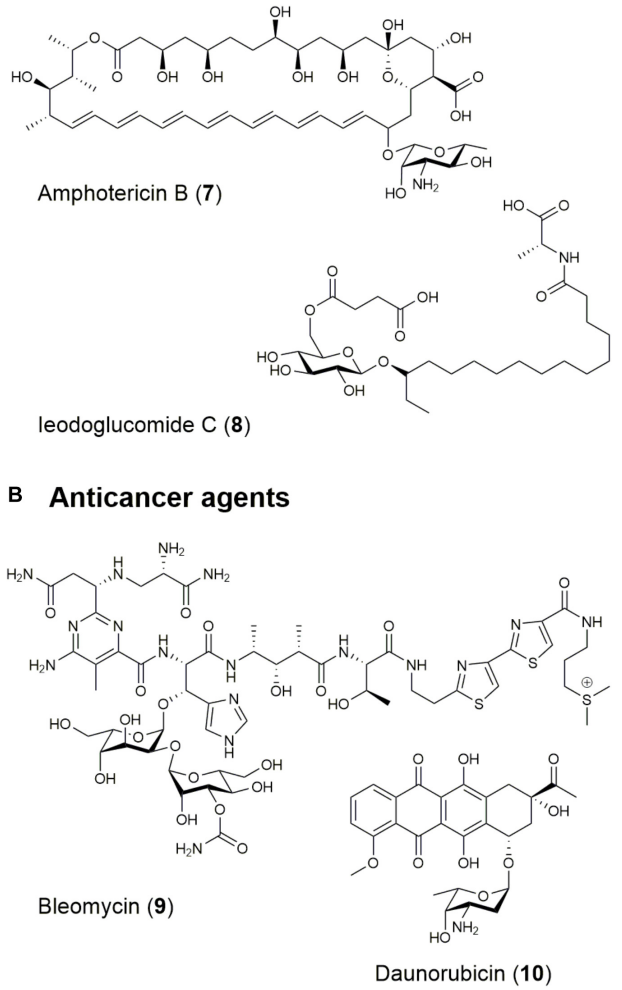

FIGURE 2 | Structures of natural products with **(A)** antifungal and **(B)** anticancer/antitumor activities.

having minimum inhibitory concentration (MIC) values below 8 mg/mL (Zida et al., 2017). The glycolipids ieodoglucomide C (8; **Figure 2** and **Table 1**) and ieodoglycolipid were isolated from the marine bacterium *Bacillus licheniformis* and exhibited antifungal activities with a 21 μg/L MIC against *Aspergillus niger*, *Rhizoctonia solani*, *Botrytis cinerea*, *Colletotrichum acutatum*, and *C. albicans* (Tareq et al., 2015). Both ieodoglucomide C and ieodoglycolipid also exhibit good antibiotic properties against *S. aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Salmonella typhi*, *E. coli* and *Pseudomonas aeruginosa* with MICs ranging from 0.01 to 0.05 μM, establishing these compounds as strong potential candidates for the development of new fungicides (Tareq et al., 2015).

Anticancer Agents

There are many microbe-derived anticancer agents that have been evaluated through clinical trials. For instance, the polyketide actinomycin was isolated from *Streptomyces parvulus* in 1940 and was the first antibiotic shown to have anticancer activity (Waksman and Woodruff, 1940; Hollstein, 1974). In particular, actinomycin D, also known as dactinomycin, is approved by FDA and has been widely used in clinical practice as an anticancer drug for treating many tumors, such as Wilms' tumor, childhood rhabdomyosarcoma, Ewing's sarcoma, and metastatic, non-seminomatous testicular cancer.

Another notable example is the therapeutic combination of the microbial product bleomycin (9; **Figure 2** and **Table 1**), the plant compound etoposide, and the synthetic agent cisplatin, which has played a significant role in increasing the cure rate for disseminated testicular cancer from 5% in 1974 to 90% in 2011 (Einhorn and Donohue, 2002; Demain and Vaishnav, 2011). Bleomycin is a glycopeptide produced by *Streptoalloteichus hindustanus* and has been used for squamous cell carcinomas, melanomas, sarcomas, testicular, and ovarian cancer, Hodgkin's and non-Hodgkin's lymphomas, and testis tumors as an anticancer agent (Demain and Vaishnav, 2011). Its derivative, blenoxane is also used clinically with other compounds against lymphomas, skin carcinomas, and tumors of the head, neck, and testicles (Demain and Vaishnav, 2011). The anthracyclines are also an important family of polyketides produced by *Streptomyces* species by iterative PKS pathways and include daunorubicin (10; **Figure 2** and **Table 1**) (Di Marco et al., 1981), epirubicin (Cersosimo and Hong, 1986), and doxorubicin (Metsä-Ketelä et al., 2008). The FDA approved the use of daunorubicin and doxorubicin for cancer therapy in the 1960s. Daunorubicin is used in the treatment of acute lymphoblastic or myeloblastic lymphoma. Meanwhile, doxorubicin is used in the treatment of breast cancer, solid tumors in children, soft tissue sarcomas, and aggressive lymphomas (Giddings and Newman, 2013).

Among numerous recent examples, rapamycin, wortmannin, and geldanamycin have been found to have antiproliferative activities during clinical use as novel chemotherapeutic agents (da Rocha et al., 2001). Rapamycin, a natural product derived from *Streptomyces rapamycinicus* has anticancer activity in addition to its immunosuppressive, anti-inflammatory, and antifungal activities (Kim et al., 2014). It performs antitumor activity on a tumor cell by hindering its proliferation, triggering apoptosis, and inhibiting angiogenesis (Law, 2005). Wortmannin is a fungal furanosteroid derivative of *Penicillium funiculosum* (Davidson et al., 2013). It has shown as an effective selective inhibitor of phosphoinositide 3-kinases (PI3Ks) and PI3K- related enzymes which are play a key role in intracellular signaling pathways (Sieber et al., 2010). A study on the proliferation and apoptosis of human breast MCF-7 cells treated with wortmannin uncovered that wortmannin shows antitumor activity by triggering apoptosis and impeding proliferation of cancer cells by suppressing PI3K/Akt signaling and NF-κB protein expression (Yun et al., 2012). Geldanamycin is a benzoquinone ansamycin antitumor compound derived from *Streptomyces hygroscopicus* var. *geldanus* (Singh et al., 2010). Geldanamycin prevents ATPase activity by binding to the heat shock protein and hindering the stability and function of oncogenic protein kinases involved in signal amplification cascade that controls proliferation and apoptosis (Singh et al., 2010). Geldanamycin and its analogs play

a key role as anticancer agent in multiple myeloma, breast, and prostate cancer (Gorska et al., 2012). Another example is epothilone, an anticancer agent produced from mycobacterium *Sorangium cellulosum*. It obstructs microtubule depolymerization thereby causing G2-M interphase arrest of the cell cycle (Molnar et al., 2000). There are also marine microbial natural products that have anticancer activities, such as dolastatin, which is originated from cyanobacteria of the genera *Symploca* and *Lyngbya* (Simmons et al., 2008).

Immunosuppressive Agents

Rapamycin (also known as sirolimus) (11; **Figure 3** and **Table 1**) and FK506 (tacrolimus) (12; **Figure 3** and **Table 1**) are microbial natural products with immunosuppressive properties. Rapamycin blocks the proliferation of most cell types in response to activation by IL-2, IL-3, platelet-derived growth factor, epidermal growth factor, and insulin (Vezina et al., 1975). Rapamycin also exhibits synergism with other immunosuppressants, such as cyclosporin, to significantly reduce kidney toxicity and acute renal allograft rejection (Yoo et al., 2017). This compound has been developed to coat coronary stents and prevent organ transplant rejection and lymphangioleiomyomatosis; it was approved by the FDA for wider use in 1999 (Mann, 2001). In addition to its immunosuppressive activity, rapamycin possesses several other biological activities, including antitumor, neuroprotective/neuroregenerative, antineoplastic, and lifespan extension activities (Law, 2005; Pan et al., 2008; Yoo et al., 2017).

FK506 is also an immunosuppressive drug and was first discovered in soil samples containing *Streptomyces tsukubaensis* and several other *Streptomyces* species (Tanaka et al., 1987). FK506 is used to minimize organ rejection and to induce immunosuppression via calcineurin inhibition and interruption of T cell activation pathway (Migita and Eguchi, 2003). It has been demonstrated to be more effective than cyclosporin and non-toxic in low doses (Demain, 2014). The discovery of its immunosuppressive activity led to its use in heart, liver, and kidney transplants with overwhelming success (Demain, 2014). Like rapamycin, FK506 possesses various biological activities, including antifungal, anti-inflammatory, neuroprotective, and neuroregenerative activities (Ban et al., 2016).

Anti-inflammatory Agents

Some natural products also have anti-inflammatory activities. FK506 has shown efficacy in the treatment of refractory rheumatoid arthritis, a chronic inflammatory disease (Migita and Eguchi, 2003). Rapamycin also inhibits the inflammatory response after spinal cord injury by diminishing the activation and proliferation of inflammatory cells and the expression of inflammatory cytokines, thereby reducing secondary injury in the spinal cord and providing a neuroprotective effect (Song et al., 2015). Recently, strepsesquitriol, isolated from *Streptomyces* sp. SCSIO 10355, has been found to have anti-inflammatory activity through the inhibition of tumor necrosis factor-α production in lipopolysaccharide-activated macrophages (Yang et al., 2013). Salinamides A and B from marine *Streptomyces* sp. CNB-091 also displayed potent topical anti-inflammatory activity through a phorbol ester-induced mouse ear edema assay (Trischman et al., 1994). One study evaluated 7 peptides found in the *Faecalibacterium prausnitzii* supernatant, all belonging to a protein named microbial anti-inflammatory molecule (Breyner et al., 2017). These peptides were able to inhibit the NF-κB pathway *in vitro* and showed anti-inflammatory properties *in vivo* in a dinitrobenzene sulfate-induced colitis model (Breyner et al., 2017).

Biofilm Inhibitory Agents

Parasitic microorganisms adhere to solid surfaces and form layers of a complex polysaccharide matrix called a biofilm that confers resistance against antibiotics as wells as inflicts significant chronic bacterial infections (Singh et al., 2017). Analogs of 5-benzylidene-4-oxazolidinones are small molecules derived from marine natural products. These molecules inhibit 89% of biofilm formed by MRSA at 0.78 μM and disperses pre-formed biofilms at 4.7 μM (Edwards et al., 2017). A synthetic library of 2-aminoimidazole triazoles was able to successfully inhibit 94% of biofilm formation in *Acinetobacter baumannii* and MRSA at 100 μM (Rabin et al., 2015). Another recent example is cahuitamycins A-C (13; **Figure 3** and **Table 1**) derived from the marine bacterium *Streptomyces gandocaensis*. Cahuitamycins have been evaluated as inhibitors of *A. baumannii* biofilms and it has been found that cahuitamycin C shows half maximal inhibitory concentration (IC_{50}) at 14.5 μM. Modifications of cahuitamycins through selective mutasynthesis have yielded cahuitamycins D and E with an increased the potency of antibiofilm activity against *A. baumannii* (Park et al., 2016). The FDA-approved antitumor agent actinomycin D has also significant biofilm inhibitory activity against methicillin resistant- and sensitive-strains of *S. aureus* (Fracchia et al., 2010; Lee et al., 2016). In addition to bacterial biofilm, fungal biofilm associated with *Candida* pathogens is responsible for serious *C. albicans* infections linked to biofilm formation on medical devices. One study showed that *Lactobacillus* biosurfactants displayed high anti-adhesive biofilm formation properties against *C. albicans* and also prevented biofilm formation of *L. monocytogenes*, *Salmonella arizonae*, *E. coli*, and *S. aureus* (Fracchia et al., 2010).

Others

Natural products can also act as antiparasitic agents. The avermectins (14; **Figure 3** and **Table 1**) and the derivative ivermectin have shown antiparasitic activity by significantly lowering the incidence of onchocerciasis and lymphatic filariasis (Shen, 2015). Spinosad and milbemycin also have insecticidal activity. Spinosad is a combination of spinosyn A and D, which are both produced by *Saccharopolyspora spinosa* and have insecticidal activity against livestock external parasites via the disruption of nicotinic acetylcholine receptors (Sanchez et al., 2012). Milbemycin is an isolated fermentation product of *S. hygroscopicus* subsp. *aureolacrimosus* that acts as an insecticide and acaricide with GABAergic activity on the post-synaptic membranes of the inhibitory motor neurons of mites and arthropods through hyperpolarization and impeding neuronal

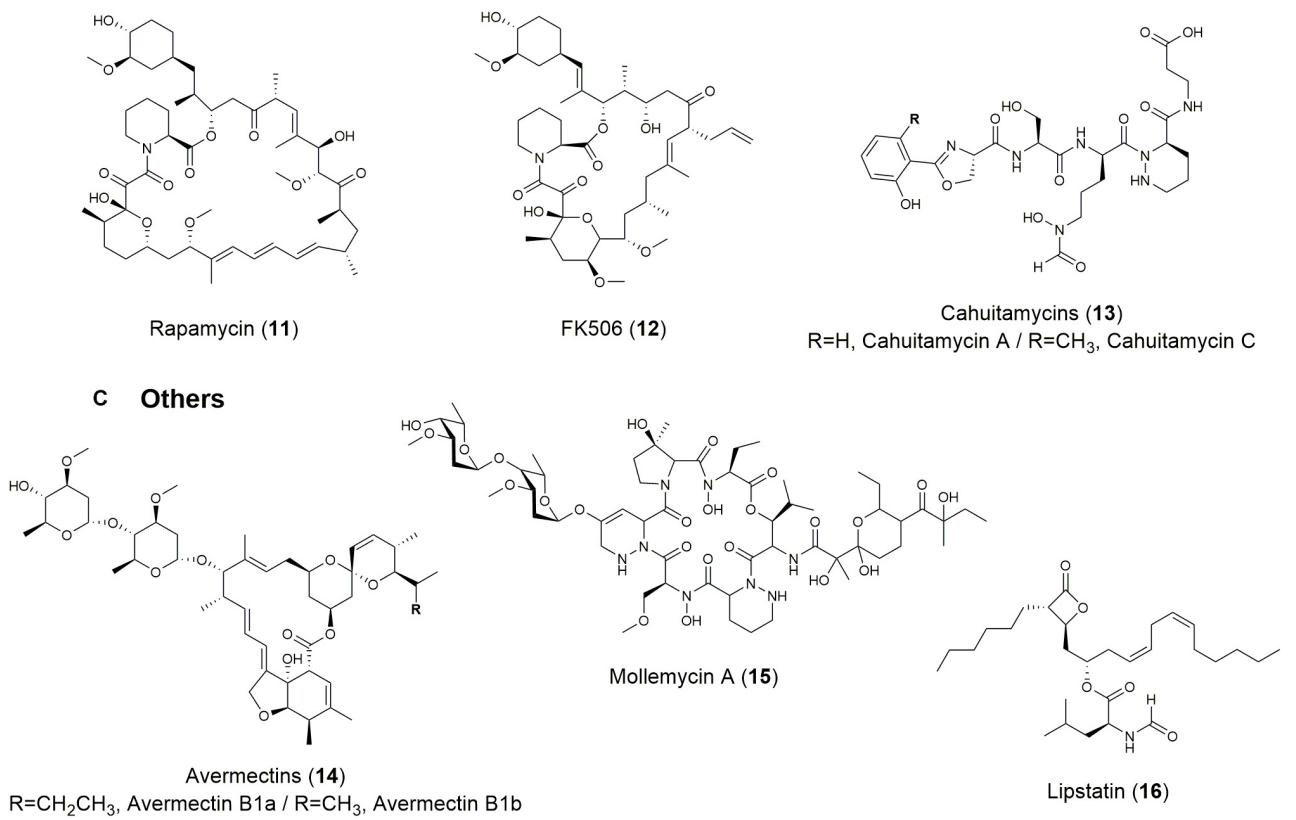

FIGURE 3 | Structures of natural products with **(A)** immunosuppressive/anti-inflammatory, **(B)** biofilm-inhibitory, and **(C)** other activities.

signal transduction mechanisms (Copping and Duke, 2007). Mollemycin A 20 (15; **Figure 3** and **Table 1**) is a first-in-class glycol-hexadepsipeptide-polyketide from a *Streptomyces* sp. and has antibacterial properties against certain Gram-positive and Gram-negative bacteria, as well as extremely potent antimalarial activity against drug sensitive and MDR *Plasmodium falciparum* clones (Blunt et al., 2016). Microbial natural products also function as enzyme inhibitors. Lipstatin (16; **Figure 3** and **Table 1**) is a pancreatic lipase inhibitor produced by *Streptomyces toxytricini* that is used to combat obesity and diabetes by interfering with the gastrointestinal absorption of fat (Weibel et al., 1987). Lipstatin contains a beta-lactone structure that is likely responsible for irreversibly binding to the active site of lipase (Sanchez et al., 2012).

Biological Activity of Microbial Biologics

Since Humulin® (**Figure 4A**) became the first recombinant biopharmaceutical as a treatment for diabetes (Johnson, 1983), additional FDA-approved microbial biologics have been produced by *E. coli*. Somatrem (Protropin®) and somatropin (Humatrope®) are used to treat children with growth hormone deficiency (Baeshen et al., 2015; Sanchez-Garcia et al., 2016). Another biopharmaceutical produced from *E. coli* is pegloticase (Krystexxa®) for the treatment of chronic gout and interferon (IFN) α-2b (Intron®A; **Figure 4B**) for the treatment of certain types of genital warts, malignant melanoma, hairy cell leukemia, follicular lymphoma, Kaposi's sarcoma, and chronic Hepatitis B or C (Baeshen et al., 2015; Sanchez-Garcia et al., 2016). Top selling biopharmaceuticals of 2015 from microorganisms include insulin glargine (Lantus®) derived from *E. coli*, which functions as an insulin analog, and the pneumococcal vaccines (Prevnar®family) derived from *S. pneumoniae* and *Corynebacterium diphtheriae* (Jozala et al., 2016; Sanchez-Garcia et al., 2016). Biopharmaceuticals are also utilized for their antitumoral properties, such as the cytokines filgrastim (Neupogen®) and granulocyte colony stimulating factor pegfilgrastim (Neupeg®; **Figure 4C**), which are both derived from *E. coli*. Flgrastim stimulates hematopoiesis for bone marrow transplantation and cancer chemotherapy-induced neutropenia, whereas pegfilgrastim stimulates the differentiation, proliferation and activation of neutrophilic granulocytes for cancer chemotherapy-induced neutropenia (Sanchez-Garcia et al., 2016). Recombinant human interleukin-3 (hIL-3; **Figure 4D**) protein is a cytokine that regulates the differentiation and proliferation of the various cells of the immune system (Hercus et al., 2013). The hIL-3 protein is derived from *B. subtilis*, *B. licheniformis*, and *E. coli* and has utility as a laboratory reagent in hematology for cell cultures, differentiation studies and

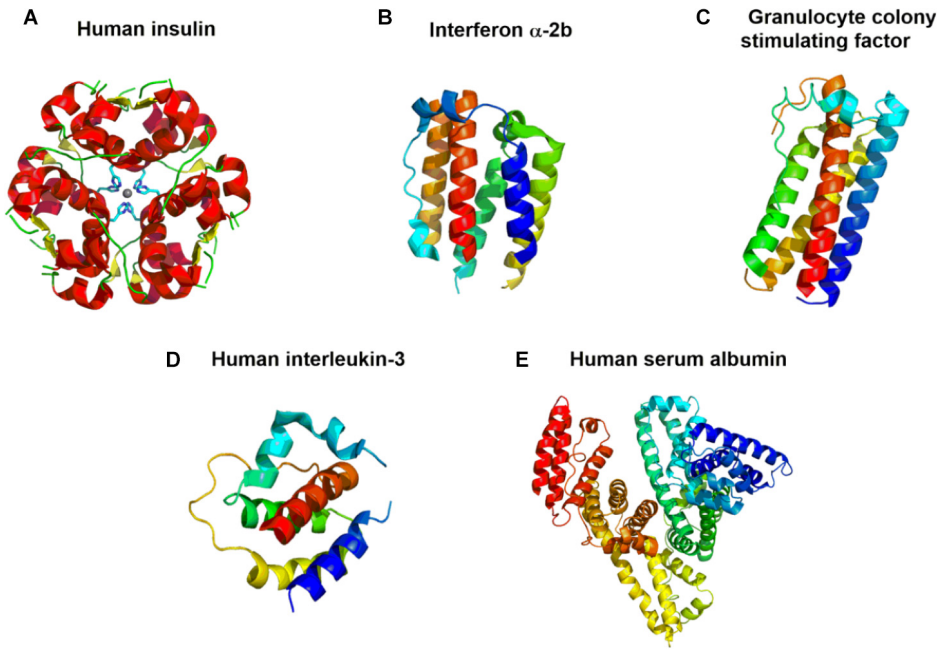

FIGURE 4 | Crystal structures of **(A)** recombinant human insulin (Humulin®) (PDB 4F0N) (Favero-Retto et al., 2013); **(B)** interferon (IFN) α-2b (PDB 3SE3) (Thomas et al., 2011); **(C)** granulocyte colony growth factor pegfilgrastim (Neupeg®) (PDB 1HRG) (Hill et al., 1993); **(D)** human interleukin-3 (PDB 5UV8) (Broughton et al., 2018); and **(E)** human serum albumin (Recombumin®and Albucult®) (PDB 1AO6) (Sugio et al., 1999). The models are colored according to the sequence by a rainbow color from the N-terminus (blue) to the C-terminus (red).

functional assays. It has shown that hIL-3 has potential in treating bone marrow failure, hematological malignancies, and can support engraftment after bone marrow transplantation (Westers et al., 2006). In addition, recombinant *Pfs48/45* is a disulfide-rich malaria transmission-blocking vaccine produced by *L. lactis* that provides immunization against malaria from *P. falciparum* (Song et al., 2017).

Recombivax is produced by *S. cerevisiae* and can prevent infection of all known subtypes of the Hepatitis B virus (Sanchez-Garcia et al., 2016). Some examples of currently approved protein therapeutics derived from yeast include human serum albumin (Recombumin® and Albucult®; **Figure 4E**), human insulin (Actrapid®) and primary immunization for infants born of Hepatitis B virus (HBV) surface antigen (Pediarix®), all of which are obtained exclusively from *S. cerevisiae* (McAleer et al., 1984; Ballance, 1999; Nielsen, 2013; Nandy and Srivastava, 2018). Recombinant human serum albumin is utilized to increase the shelf life of protein drugs by preventing physical and chemical degradation. Actrapid® is used to treat diabetes, and subcutaneous injections of Pediarix is designed for immunization against diphtheria, tetanus, pertussis, poliomyelitis, and infection caused by all known subtypes of HBV (Nandy and Srivastava, 2018). Ecallantide (Kalbitor®) is an FDA-approved recombinant peptide produced by *Pichia pastoris* for the treatment of hereditary angioedema (Sheffer et al., 2011). Additionally, anakinra (Kineret®) was approved in 2001 in the United States for rheumatoid arthritis (Baeshen et al., 2015). Anakinra is expressed in *E. coli* and functions as an IL-1 receptor antagonist that is effective and safe for patients with systemic-onset juvenile idiopathic arthritis, adult-onset Still's disease, hereditary autoinflammatory syndromes, and Schnitzler's syndrome (Kalliolias and Liossis, 2008; Jozala et al., 2016).

MICROBIAL CELL FACTORIES

Selecting a suitable host strain is one of the most important aspects in the design of natural product and recombinant protein bioprocesses. We will review the characteristics of the microbial strains used to produce natural products and biologics in this section. We will also present the tools and strategies that facilitate engineering of the hosts as microbial cell factories for the production of biopharmaceutical compounds (**Table 2**).

Gram-Negative Bacteria

Escherichia coli

Escherichia coli has been seen as one of the optimal systems for the production of natural products because it is easily manipulated, highly productive, there is an availability of genetic tools to use with it and there is a deep knowledge of its physiology. Artemisinin, a sesquiterpene lactone endoperoxide from *Artemisia annua* L. plants, has strong antimalarial activity against the multi-drug resistant parasite *P. falciparum* (Abdin et al., 2003). Yet the synthesis of artemisinin is costly and low yields are isolated from the natural plant source. Researchers reported the production of approximately 24 mg/L of amorpha-4,11-diene (amorphadiene), an artemisinin

TABLE 2 | Comparison between different microbial host systems for production of recombinant proteins and natural products.

Microbial hosts	Advantages	Disadvantages	Compounds	References
Gram-negative				
Escherichia coli	• Fast growth • Simple culture procedures • Cost-effective • High versatility of the enterobacterium and its associated systems	• Lack of post-translational modifications (PTMs) • Risk of translational errors due to the presence of a large number of rare codons • Expensive and often challenging purification process	• Recombinant human insulin • Artemisinin • Erythromycin A • Somatrem • Somatropin • Pegloticase • Insulin glargine • Pneumococcal vaccines • Filgrastim • Pegfilgrastim • Human serum albumin • Hepatitis B virus immunization • IFN α-2b • IL-6	Johnson, 1983; Abdin et al., 2003; Chang et al., 2007; Zhang et al., 2010; Ferrer-Miralles and Villaverde, 2013; Baeshen et al., 2015; Jozala et al., 2016; Sanchez-Garcia et al., 2016
Gram-positive				
Lactococcus lactis	• Simplified downstream purification processes • Absence of endotoxins or unwanted glycosylation of proteins • Generally recognized as safe (GRAS) • Lack of secreted heterologous proteins degradation • Nisin-controlled gene expression system • Heterologous protein delivery in foodstuff or in the digestive tract	• Per liter secretion generally less robust than *Bacillus* sp. • AT-rich codon usage and/or the distribution of rare codons	• Nisin A • *Pfs48/45* • Enterocin A • Pediocin PA-1 • IL-2 • IL-6 • Peanut allergen • Tetanus toxin fragment C • Transforming growth factor-β1	Steidler et al., 1998; Drouault et al., 2000; Martínez et al., 2000; Le Loir et al., 2005; Mierau and Kleerebezem, 2005; Glenting et al., 2007; Morello et al., 2008; Li and Vederas, 2009; Linares et al., 2010; Gyawali and Ibrahim, 2014; Bermúdez-Humarán et al., 2015; Li et al., 2015; Song et al., 2017
Streptomyces sp.	• Rapid growth • Abundant supply of secondary metabolite precursors • Ability to produce natural products. • Efficient protein secretion system such as Sec pathway and twin-arginine-translocation (Tat) pathway • Well-developed genetic manipulation	• Forms pellets or clumps • Low protein yield	• Streptomycin • Pikromycin • Kanamycin • Nystatin • Anthracyclines • Rapamycin • FK506 • Strepsesquitriol • Salinamides A and B • Cahuitamycin • Actinomycin D • Milbemycin • Mollemycin A • TNF α • hIL-10 • Streptokinase • IL-1β • IFN-α1 • Transforming growth factor-α • IL-2 • IFN-α2b • Tetracycline • Daptomycin • Chloramphenicol	Stanley and English, 1965; Kaslow et al., 1994; Trischman et al., 1994; Mann, 2001; Jung et al., 2006; Copping and Duke, 2007; Park et al., 2008, 2016; Vrancken and Anne, 2009; Fracchia et al., 2010; Anné et al., 2012; De Lima Procópio et al., 2012; Sanchez et al., 2012; Yang et al., 2013; Kim et al., 2015; Blunt et al., 2016; Jozala et al., 2016; Gao et al., 2017

(Continued)

TABLE 2 | Continued

Microbial hosts	Advantages	Disadvantages	Compounds	References
Bacillus sp.				
	• Outstanding fermentation properties and protein production yield (20–25 g per liter) • Completely free toxin production • Flexibility for genetic engineering • Presence of proteome secretory pathway	• Primarily used in Enzyme production. • Plasmid instability • Presence of proteases: leads to difficulty in the production of recombinant proteins.	• Ieodoglucomide C • Ieodoglycolipid • Bacillomycin D and L • Alkaline cellulose • Alkaline protease • Alkaline α-amylase • hIL-3 • Fengycin • IL-1β • IFN-α2 • Staphylokinase • Iturins • Surfactin	Palva et al., 1983; Peypoux et al., 1984; Bellini et al., 1991; Kim et al., 2001; Westers et al., 2006; Deleu et al., 2008; Chang et al., 2011; Van Dijl and Hecker, 2013; Wang T. et al., 2015; El-Hossary et al., 2017
Fungi/yeast				
Saccharomyces cerevisiae				
	• Fast growth rate • Technically practical • Cost-effective • Ability to generate post-translational modification as *O*-linked glycosylation, phosphorylation, acetylation, and acylation • Advanced fermentation science	• *N*-linked glycosylation patterns differ from higher eukaryotes • Lack some required precursor pathways • Codon usage is biased toward A + T	• Human serum albumin • Recombinant human insulin • Hepatitis B virus immunization • Artemisinic acid • Paclitaxel • hIL-6 • Insulin aspart • *Pfs25* • Sapogenin • Saponin	McAleer et al., 1984; Guisez et al., 1991; Kaslow et al., 1994; Ballance, 1999; Ferrer-Miralles et al., 2009; Nielsen, 2013; Paddon et al., 2013; Baeshen et al., 2014; Ding et al., 2014; Meehl and Stadheim, 2014; Moses et al., 2014; Kung et al., 2018; Nandy and Srivastava, 2018
Aspergillus sp.				
	• GRAS status • Tolerate extreme cultivation conditions • Degrade and utilize diverse biopolymers, allowing cultivation on renewable resources • Major Source of citric acid production	• Production of mycotoxins (alpha toxins) • Many host proteases • Freely dispersed filaments or highly compact pellets formed during submerged fermentations	• Immunoglobulin G1(κ) • Antibodies and Fab′ fragment • Bicoumanigrin • Aspernigrin B • Lactoferrin • Enniatin • Human IL-2 • Human IL-6 • Phytase • L-asparaginase • Lovastatin • Tryptostatin B	Gaffar and Shethna, 1977; Carrez et al., 1990; Hiort et al., 2004; Papagianni, 2004; Ward et al., 2004; Grimm et al., 2005; Maheshwari, 2006; Pel et al., 2007; Maiya et al., 2009; Meyer et al., 2011; Cragg and Newman, 2013
Hansenula polymorpha				
	• GRAS status • Combined genetic manipulations, low cost screening. • Efficient fermentation properties, and protein modification • Ability to use and grow on methanol, glucose, or glycerol as its primary carbon sources • Thermo-tolerant	• The use of methanol creates hazardous conditions in lab use • Hyperglycosylation of heterologous products • Can lead to production instabilities due to sequence repetition on vector.	• IFNα-2a • Phytase • IL-6 • Human serum albumin • Human hemoglobin • HBV L-protein • Hepatitis B surface antigen	Janowicz et al., 1991; Gellissen et al., 1992; Hollenberg and Gellissen, 1997; Cox et al., 2000; Heijtink et al., 2002; Müller et al., 2002; Böer et al., 2007; Kunze et al., 2009; Celik and Calik, 2012

precursor, by the expression of a codon-optimized synthetic amorphadiene synthase gene and the mevalonate pathway from *S. cerevisiae* in *E. coli*. Additionally, after further processing modifications and optimal conditions, they were able to produce 105 mg/L of artemisinic acid (Chang et al., 2007). However, there are some obstacles and limitations with *E. coli* as a dominant host in natural product biosynthesis. *E. coli* requires extensive genetic manipulation and lacks native natural product biosynthetic machinery and/or precursors. An example is phosphopantetheinyl transferase, which is responsible for the activation of the carrier protein domains of the PKSs and NRPSs. This enzyme must be introduced into *E. coli* to support of natural product biosynthesis (Zhang M.M. et al., 2016). There have been efforts to overcome these hurdles, such as the production of erythromycin A and its derivatives in the engineered *E. coli* strain (Zhang et al., 2010). The study generated two analogs through directed manipulation of polyketide biosynthesis in which variations were made to the deoxyerythronolide B synthase (DEBS) 1 and DEBS3 enzymes in order to utilize the multi-catalytic capability of the modular polyketide synthase (Zhang et al., 2010).

Escherichia coli has also been the pioneering host for recombinant protein production. To date, *E. coli* continues to be the first-choice microorganism for manufacturing recombinant proteins at laboratory and industrial scales. Its success is mostly due to its fast growth, simple culture procedures, cost-effectiveness, unusually high versatility, and the associated systems that make it adaptable to varying production demands (Ferrer-Miralles and Villaverde, 2013; Sanchez-Garcia et al., 2016). From 2004 to 2013, 24% of the biopharmaceuticals approved by the FDA and the European Medicines Agency were derived from *E. coli* (Baeshen et al., 2014). Currently, biopharmaceuticals produced from *E. coli* are used in the treatment of diabetes, growth hormone-deficiency in children, leukemia, gout, and many other therapeutic indications as previously discussed in Section "Biological Activity of Microbial Biologics" (Baeshen et al., 2015). A major concern when using *E. coli* as a production platform is the lack of post-translational modifications (PTMs) present in most eukaryotic proteins; lacking PTMs can lead to protein products being insoluble, unstable, or inactive (Ferrer-Miralles et al., 2009). However, it is possible to add synthetic PTMs to generate versions of the protein that are more stable than the original naked product (Ferrer-Miralles et al., 2009). Examples of this include pegylated products, like human growth hormone, stimulating factor, IFNs α-2a and α-2b, (Ferrer-Miralles et al., 2009). Additionally, there is a risk of translational errors due to the presence of a large number of rare codons that appear in human genes that are different from those occurring in *E. coli* genes. Even at low levels, these errors may cause an impact on the tertiary structure, premature termination of protein synthesis or amino acid misincorporation which results in low protein expression (Gustafsson et al., 2004). One strategy to bypass the issue with codon bias is to synthesize the whole human gene based on codon usage in *E. coli* through site-directed mutagenesis, which is currently a preferred method; however, it is limited by the high cost of production and time consumption (Sørensen and Mortensen, 2005). An alternative method that is less time consuming utilizes the co-transformation of *E. coli* strains with a plasmid(s) containing a gene encoding the tRNA cognate to the rare codons (Dieci et al., 2000). Increasing the copy number allows for *E. coli* to be manipulated to match the codon usage frequency in heterologous genes (Dieci et al., 2000). Currently, there are numerous commercial *E. coli* strains available that harbor plasmids containing gene sequences encoding the tRNA for rare codons, such as BL21(DE3) CodonPlus-RIL, BL21(DE3) CodonPlus-RP and Rosetta (DE3) (Baeshen et al., 2015). Another common problem associated with recombinant protein expression in *E. coli* involve inclusion body formation, which refers to insoluble and inactive protein aggregates (Hartley and Kane, 1988). *E. coli* producing recombinant proteins have the ability to assemble in cells and form conglomerates of inclusion bodies as well as result in erroneous protein folding which hinder the extraction of proteins directly from the cell leading to costly purification of proteins (Zweers et al., 2008). Inclusion bodies formed from lack of proportion between protein solubilization and aggregation can be resolved by combining the desired protein with a solubility enhancer fusion partner acting as an intrinsic chaperone in order to ensure the production of soluble recombinant proteins (Rosano and Ceccarelli, 2014). The fusion of maltose-binding protein to polypeptides such as human tissue inhibitor of metalloproteinase and p16 improved their solubility significantly in *E. coli* (Kapust and Waugh, 1999).

Gram-Positive Bacteria

Lactococcus lactis

Lactococcus lactis is becoming an attractive alternative in genetic engineering for the production of various recombinant proteins. Unlike *E. coli*, which uses intracellular production strategies that involve expensive and often challenging purification processes, *L. lactis* utilizes extracellular secretion system. This is because *L. lactis* has a monolayer cell wall that allows direct secretion into the extracellular environment (Schneewind and Missiakas, 2012). The presence of exported proteases such as HtrA in *L. lactis* contributes to recombinant protein production by minimizing the destruction of heterologous proteins in the medium (Morello et al., 2008; Song et al., 2017). Additionally, *L. lactis* does not generate undesired glycosylation of protein, is generally recognized as safe (GRAS), does not produce endotoxins, and has probiotic properties (Singh et al., 2018). Another advantage of *L. lactis* includes a lack of inclusion body formation (Theisen et al., 2017). There is a diverse selection of cloning and inducible expression vectors available for use with this host that are compatible with large-scale upstream and downstream processes (Song et al., 2017).

Lactococcus lactis has been used for centuries in the fermentation of food, especially in cheese, yogurt, and sauerkraut because of its production of nisin (Song et al., 2017; Singh et al., 2018). Beyond the food industry, lactic acid is used as an emulsifier and moisturizing agent in the cosmetic industry and as an important raw material in the pharmaceutical industry (Papagianni, 2012). The *L. lactis* host has also been chosen after researchers had unsuccessfully attempted to obtain correct conformation using a variety of other

prokaryotic and eukaryotic recombinant protein expression systems. *L. lactis* has multiple advantages over *E. coli* for expression of 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (*Pfs*) recombinant proteins, including the following: (1) codon-optimization of the recombinant gene is not necessary to achieve successful expression in *L. lactis*; (2) the recombinant protein is secreted into the *L. lactis* culture supernatant, which results in easier and less expensive down-stream processing, and (3) there is no lipopolysaccharide contamination in *L. lactis* expression (Singh et al., 2018). *L. lactis* has been used in the successful production of recombinant *Pfs48/45*, a vaccine candidate against *P. falciparum* (Song et al., 2017). GMZ2, a recombinant fusion protein expressed in *L. lactis*, is also a malaria vaccine candidate that has been shown to elicit high levels of active IgG antibodies with inhibitory activity against a broad range of *P. falciparum* strains (Jepsen et al., 2013). A recent study concluded phase 2 trial of GMZ2 adjuvanted with aluminum hydroxide in a cohort of 1,849 children revealed GMZ2 as well tolerated with modest efficacy (Sirima et al., 2016). Not only is *L. lactis* being utilized for the production of recombinant proteins for vaccines, but the host is also being tested as a factory for antigen production, allowing the bacteria to function as live vaccines. Using *L. lactis* as a vaccine carrier is beneficial because it can induce both mucosal and systemic immune responses, has adjuvant properties and is free from the risks associated with the use of conventional attenuated live pathogens, such as *Salmonella* species and *Mycobacterium* species (Song et al., 2017). However, while *L. lactis* has been studied against an array of antigens from various pathogens, there is no current live vaccines under clinical trial which may be due to a lack of containment strategies (Bahey-El-Din, 2012). Without a plan for containment, studies on live *L. lactis* vectors risk the chance of proliferation and dispersion. An additional limitation of AT rich *L. lactis* as a cell factory is due to codon usage as well as distribution of rare codons to express heterologous genes (Mierau and Kleerebezem, 2005).

Streptomyces Species

Another major species that has shown promise as a cell factory through its wide production of natural products and biologics is *Streptomyces*. This Gram-positive bacterium has been studied for more than 70 years and has proven to be of great use in biotechnology due to its ability to produce natural products acting as antibiotics, anticancer agents, and immunosuppressants (Yoo et al., 2015). Some examples include tetracycline, daptomycin, and chloramphenicol (De Lima Procópio et al., 2012). There are many species of *Streptomyces* currently to produce various natural products and biologics. Among the *Streptomyces* species, *Streptomyces coelicolor*, *Streptomyces lividans*, *Streptomyces albus*, and *S. venezuelae* are favored heterologous hosts to produce specialized metabolites due to the relative ease of their genetic manipulation, the availability of their genome sequences, and the abundant supply of their natural substrates (Park et al., 2010). *Streptomyces* has also been used to produce a wide array of heterologous proteins of both eukaryotic and prokaryotic origin (Gilbert et al., 1995) because *Streptomyces* has well-developed genetic manipulation and fermentation technologies as well as efficient protein secretion systems such as the secretory (Sec) pathway and the twin-arginine-translocation (Tat) pathway (Hamed et al., 2018). The Sec-pathway catalyzes the translocation of unfolded proteins while the Tat pathway allows for the export of folded proteins across the cytoplasmic membrane (Natale et al., 2008). Tumor necrosis factor (TNF) α and human interleukin (hIL) 10 are able to be expressed in both the Sec- and Tat-pathways (Schaerlaekens et al., 2004). In particular, *S. lividans* could be the ideal *Streptomyces* host due to limited restriction-modification systems and low endogenous protease activity (Nakashima et al., 2005). Streptokinase (Pimienta et al., 2007), transforming growth factor-α (Taguchi et al., 1995), IL-2 (Bender et al., 1990) and many other proteins have been successfully expressed and secreted from *S. lividans.* However, aside from its efficient secretory pathways, when in culture, *Streptomyces* grows as mycelial networks, leading to the formation of pellets or clumps (Van Dissel et al., 2015). These pellets are unappealing from an industrial standpoint because of mass-transfer problems, slow growth, and culture heterogeneity which leads to lower product yield (Van Dissel et al., 2015).

Bacillus Species

Bacillus species are some of the most popular species used in enzyme production. It accounts for roughly 50% of enzymes market within the industrial sphere (Barros et al., 2013). Certain species, like *B. subtilis*, *Bacillus amyloliquefaciens*, and *B. licheniformis* are favored because of their outstanding fermentation properties, high protein production yield, and their completely toxin-free production (Van Dissel et al., 2015). The fermentation capacity of *Bacillus* species in acid, neutral, and alkaline pH ranges in addition to thermophiles accounts for the prolific production of enzymes that have desirable temperature, pH, and stability, which makes them appealing for specific use in various industries (Schallmey et al., 2004). *Bacillus* species are known for their production of iturins and fengycin which have antifungal activity as well as surfactin for its function as a surfactant (Wang T. et al., 2015).

Among these species, *B. subtilis* is the most widely studied due to (1) its flexibility during genetic engineering, (2) its naturally high secretory capacity, (3) its ability to produce valuable antibiotics, such as bacillomycins D-L and bacitracin, and (4) its ability to produce enzymes, such as stable alkaline cellulase, alkaline protease and alkaline α-amylase. It may also elicit better folding conditions, leading to the prevention of inclusion bodies (Peypoux et al., 1984; Van Dijl and Hecker, 2013). In addition, its ability to adapt to varying environmental conditions as well as its classification as toxic free GRAS has contributed tremendously to its success in the industrial platform (Baysal and Yıldız, 2017). *B. subtilis* as an endotoxin free host amplified its utilization in the production of sterile recombinant and therapeutic proteins expression as compared to *E. coli* which could have potential contamination due to the lipopolysaccharide endotoxins (Wang et al., 2013). For instance, *B. subtilis* and *Bacillus megaterium* were the preferred hosts over *E. coli* in the production of bioengineered heparin in order to diminish toxin contamination (Wang et al., 2013). Moreover, *B. subtilis* is able to produce high yield in enzyme as it secretes the enzymes

straight into the fermentation medium due to the absence of outer membrane which allows easy recovery of purified proteins from the medium into their active form (Zweers et al., 2008). It has the capacity to secrete about 20–25 g/L of enzymes into the medium (Schallmey et al., 2004). Enzymes produced by *B. subtilis* has a wide variety of applications ranging from pharmaceutical, leather, detergent, food, and waste management industries (Singh et al., 2016).

Aside from enzyme production, cytokines like hIL-3 have been produced by *B. subtilis* and *B. licheniformis*. The production of hIL-3 has been tested in various host organisms, including *E. coli*. However, the production of IL-3 within other organisms has often exhibited problems, such as insolubility or the degradation of produced hIL-3. This led to the use of *B. licheniformis* and *B. subtilis* to minimize such complications. The production of hIL-3 in *B. licheniformis* was engineered to lack four C-terminal residues, resulting in a fully active hIL-3 protein. However, residual proteolytic degradation of some hIL-3 still occurred, leading to use *B. subtilis* to achieve complete folding and full biological activity of hIL-3 (Westers et al., 2006).

Among the *Bacillus* species, *Bacillus thuringiensis* is best known for being widely used within the agricultural industry due to its insecticidal properties through its production of parasporal crystals during the stationary phase of its growth cycle (Höfte and Whiteley, 1989; Schnepf et al., 1998). Upon ingestion, the parasporal crystals are solubilized in the midgut of insects, resulting in the release of protoxin proteins known as δ-endotoxins, leading to the formation of pores throughout the cell membrane (Höfte and Whiteley, 1989; Gill et al., 1992). Parasporal proteins also have selective cytotoxicity against liver and colon cancer cells while leaving normal cells unharmed (Ito et al., 2004).

However, the use of *Bacillus* has been restricted to mainly enzyme production and non-recombinant protein therapeutics, which may be due to the lack of associated expression vectors, plasmid instability and the presence of native proteases (Westers et al., 2004). Despite *B. subtilis* success as the industrial workhorse, it has its drawbacks in the production of heterologous proteins. Heterologous protein yield could diminish when using the *Bacillus* as a host due to the proteolytic destruction of foreign protein by host secreted extracellular proteases (Nijland and Kuipers, 2008). Efforts have been made to improve the production of heterologous protein by manipulating the expression of proteins involved in the post translocation phase resulting in amplified levels of heterologous protein secretion (Vitikainen et al., 2005). In contrast to *E. coli*, the absence of distinguished and controllable promoters in *B. subtilis* interferes with successful expression of heterologous genes resulting in inefficient production of heterologous proteins (Schallmey et al., 2004).

Yeast/Fungi

Saccharomyces cerevisiae

As with *E. coli*, *S. cerevisiae* has been extensively used for the production of recombinant human insulin since the early 1980s, and it currently produces half of the world's supply of insulin (Meehl and Stadheim, 2014). *S. cerevisiae* is preferred because it is also cost-effective, fast growing, technically practical, and is amenable to large-scale fermentation in bioreactors. Yeast is often utilized as a cell factory when the target protein is not produced in a soluble form in prokaryotic systems or when a specific PTM cannot be produced or added to the naked product. *S. cerevisiae*, as with other yeast species, can perform many PTMs such as *O*-linked glycosylation, phosphorylation, acetylation, and acylation, which allows recombinant proteins to be expressed in a soluble, correctly folded, and functionally active form (Ferrer-Miralles et al., 2009; Baeshen et al., 2014). Some examples of currently approved protein therapeutics derived from yeast include human serum albumin, insulin, and primary immunization for infants born of HBV surface antigen, all which are obtained in *S. cerevisiae* (McAleer et al., 1984; Ballance, 1999; Nielsen, 2013; Nandy and Srivastava, 2018). However, the significant drawback to producing protein therapeutics from *S. cerevisiae* is that higher eukaryotes have a different pattern of *N*-linked glycosylation, which can decrease the half-life and cause hyper-immunogenicity, leading to less effective therapeutics (Ferrer-Miralles et al., 2009). In recent years, there have been some advances in limiting *S. cerevisiae* hypermannosylation. These yeast glycoengineering techniques involve two main stages, (1) the removal of yeast hypermannosylation and (2) the conversion to complex glycoforms containing terminal sugars, such as *N*-acetylglucosamine, galactose, or sialic acid. These recent reports on yeast *N*-glycan humanization indicate a move from the proof of concept phase to implementation (Meehl and Stadheim, 2014).

Another current area of study is the production of plant and microbe-derived secondary metabolites. Due to the structural complexity of secondary metabolites, chemical synthesis is an inefficient route for large-scale production, and fermentation is the main mode for economic commercial production of pharmaceutically useful natural products (McDaniel et al., 2001). *S. cerevisiae* could be an ideal candidate as a microbial host as it boasts relatively rapid growth, and it is accompanied by highly developed genetic tools and advanced fermentation science. Like *E. coli*, *S. cerevisiae* has been shown to be an outstanding production host for artemisinic acid, a precursor to the antimalarial agent artemisinin, with a high productivity (25 g/L) that led to the industrial production of semi-synthetic artemisinin beginning in 2013 (Paddon et al., 2013; Kung et al., 2018). Research has also produced the paclitaxel (Taxol®) precursor taxadiene (∼73 mg/L) by engineering the taxol biosynthetic genes in *S. cerevisiae* (Ding et al., 2014). Besides plant secondary metabolites, *S. cerevisiae* has generated a remarkable titer (1.7 g/L) of microbial polyketide 6-methylsalicylic acid in un-optimized shake-flask fermentations. In addition, *S. cerevisiae* has been developed as a heterologous host to express fungal cryptic BGCs and their respective metabolites. In this study, 30 *ADH2*-like promoters in *Saccharomyces* species were developed as tools for expression of 41 heterologous BGCs, 22 of which were capable of producing heterologous compounds, including novel compounds. For example, BGCs derived from basidiomycete were found to produce *N*-, *S*-bis-acylated amino acids and a leucine *O*-methyl ester with an additional polyketide chain

amidated to the amino ester (Harvey et al., 2018). However, barriers still exist to the heterologous production of complex molecules. This includes the production of polyketides in *S. cerevisiae*, as the host lacks some required polyketide precursor pathways, its codon usage is biased toward A + T (most microbial polyketide producers have high G + C genome content) and it lacks the appropriate endogenous phosphopantetheinyl transferase capable of the necessary PTMs (Mutka et al., 2006).

Aspergillus Species

Multicellular filamentous fungi, such as *A. niger* and *Aspergillus oryzae*, can also offer great potential in the production of a desired substance by fermentation due to the following reasons: (1) they are well-characterized GRAS organisms, (2) are amenable to scaled-up fermentation, (3) can be genetically engineered, (4) they are capable of secreting a high level of proteins and (5) can withstand adjustable cultivation conditions, including temperature (5–45°), pH (2–11), salinity (as much as 34%), water activity (0.92–0.98), and both nutrient rich and poor environments (Maheshwari, 2006; Meyer et al., 2011).

Aspergillus niger has been predominantly used for industrial-level production of citric acid through anaerobic fermentation process. As a weak acid, citric acid can serve as a natural preservative, flavoring agent in food and beverages, antioxidant, acidulant, pH-regulator, chelating agent or vegetable rinse, as well as comparable applications in the pharmaceutical and cosmetics industries (Cairns et al., 2018). Due to its wide variety applications, its ease of production, and economical potential of citric acid, the market of citric acid has become one of the fastest-growing regions of the food additives market due to the rising demand: according to estimations, in 2007, the market value of citric acid exceeded $2 billion in 2014 and is predicted to rise to $3.6 billion by 2020 (Show et al., 2015; Cairns et al., 2018). Phytase is another example that have produced by *A. niger* fermentation (Papagianni et al., 1999). The significance of phytase enzymes lie in its ability to interact with the nutrient rich compounds known as phytate. Phytate, or phytic acid, is a common energy source found in oilseeds, cereals, and legumes, which are used in providing nutrition to animal feeds (Schlemmer et al., 2009). Combining citric acid with phytase has also been shown to enhance phytase activity on phytate, producing greater nutrient outcomes in tested animals (Boling et al., 2000). In addition, two different humanized immunoglobulin G1(κ) antibodies and an Fab′ fragment were produced by *A. niger*, and the antibodies were successfully secreted into the culture supernatant (Ward et al., 2004). *Aspergillus* strains have been also used to produce the human iron-binding glycoprotein lactoferrin and hIL-2 with the yields of 25 and 150 mg/L, respectively (Maheshwari, 2006). Bicoumanigrin with cytotoxic activity against human cancer cell lines and aspernigrin B with neuroprotective effects have both been isolated from *A. niger* (Hiort et al., 2004). The product spectrum of *Aspergillus* species is not restricted to biologic molecules. As an example, a novel cyclic peptide compound, KK-1, with potent antifungal activity was produced in *A. oryzae* by introducing gene clusters spanning approximately 40 kb from the plant-pathogenic fungus *Curvularia clavata* into the genome of *A. oryzae*. Although the amount of KK-1 produced by the host was lower than that produced by the original producer *C. clavata*, this result indicated that a gene twice as large as the largest native gene in *A. oryzae* could be successfully expressed (Yoshimi et al., 2018). Furthermore, when the industrial fungus *A. niger* was engineered as a heterologous host, it produced high titers (up to 4,500 mg/L) of enniatin belonging to non-ribosomal peptides with antibacterial, antiviral, and anticancer activities (Richter et al., 2014).

Hansenula polymorpha

Another industrially important yeast species that has shown promise in the production of peptides is *Hansenula polymorpha* (Gellissen et al., 1992; Boer et al., 2007). *H. polymorpha* is a methylotrophic yeast species with the ability to use and grow on methanol, glucose, or glycerol as its primary carbon source (Gellissen et al., 1992). Like *S. cerevisiae* and *Aspergillus* species, *H. polymorpha*, classified as GRAS organism, does not harbor pyrogens, toxins, pathogens, or viral inclusions (Ubiyvovk et al., 2011). It is distinguished by very high cell densities in bioreactors and characterized by simple cultivation mode in inexpensive growth media. For example, *H. polymorpha* has allowed for cost-effective production of phytase through cheap carbon sources (Mayer et al., 1999). It possesses well-established genetic tools such as strong regulatory and constitutive promoters, which consequently give high product yield (Van Dijk et al., 2000). It also has thermotolerance properties, making *H. polymorpha* successful in crystallographic studies and in the production of recombinant proteins like IFN-2α, IL-6, recombinant human serum albumin, glucose oxidase, and catalase (Kunze et al., 2009; Celik and Calik, 2012). A notable feature of *H. polymorpha* is the significant growth of peroxisomes when grown on methanol which allows for high storage capacity of soluble proteins. The lack of protein modifying enzymes in the matrix of peroxisomes also provides an advantage for the development of heterologous proteins that are susceptible to proteolytic degradation (Van Dijk et al., 2000). Furthermore, the host has been used to produce L antigens found on the HBV viral envelope in attempt to produce the HBV vaccine. The L protein produced by *H. polymorpha* has increased stability in comparison to other yeast species, such as *S. cerevisiae* and *P. pastoris* (Janowicz et al., 1991). In addition to its use in vaccine production, *H. polymorpha* is also used in the production of human hemoglobin through the use of a single expression vector (Hollenberg and Gellissen, 1997). However, hyperglycosylation has been observed as a main drawback of *H. polymorpha* to produce heterologous products (Müller et al., 1998).

EFFORTS IN PRODUCT IMPROVEMENTS AND GENERATION OF NEW ANALOGS

There are multiple approaches that have been taken to advance product improvement for microbial natural products and biologics. This section will discuss efforts to combat the

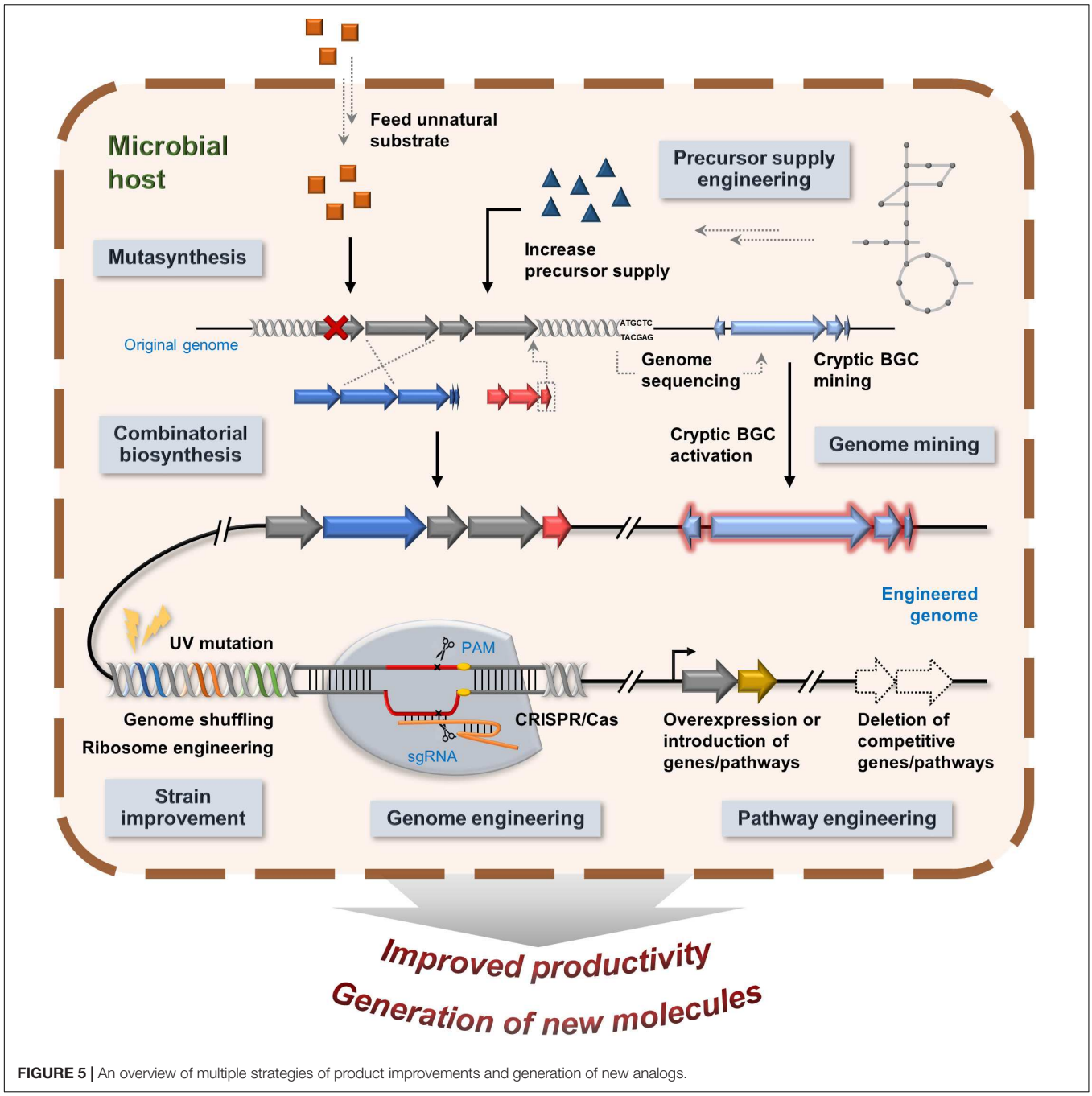

FIGURE 5 | An overview of multiple strategies of product improvements and generation of new analogs.

challenges of production of natural products and its analogs, including strain improvement, increasing precursor supply, pathway engineering, combinatorial biosynthesis, and genome mining (**Figure 5**).

Strain Improvement

Whole-genome shuffling is a process that utilizes the advantages of the multi-parental crossing allowed by DNA shuffling with the genome recombination normally associated with conventional breeding (Zhang et al., 2002). Genome shuffling has been successfully improved the titers of variety of microorganisms. For example, two strains of *Streptomyces fradiae* generated from two rounds of genome shuffling were able to produce up to a ninefold increase in antibacterial tylosin production in comparison to the initial strain (Zhang et al., 2002). Using genome shuffling in a combination of protoplast fusion, mutant strain of *S. cellulosum* $GSUV_{3-205}$ generated a 130-fold increase (104 mg/L) in production of epothilone when compared to starting strain *S. cellulosum* So0157-2 (0.8 mg/L) (Gong et al., 2007). Ribosome engineering is also a method useful in increasing secondary metabolite production titer and productivity (Sun and Alper, 2015). Studies demonstrate

that *rpoB* mutations are effective in activating silent and poorly expressed secondary metabolite biosynthetic gene clusters (BGCs) at the transcriptional level in *S. griseus*, *S. coelicolor*, and *S. erythraea* (Ochi and Hosaka, 2013). For example, the H437R mutant of *rpoB* from *S. erythraea* was screened for drug resistance and was found to have an increased production of erythromycin (Tanaka et al., 2013; Ochi et al., 2014). Another study found a 37-fold increased production of avilamycin in a recombinant *Streptomyces viridochromogenes* strain due to a mutation in ribosome protein S12 (*rps12*) acquired through a combination of gene shuffling and ribosome engineering (Lv et al., 2013).

Engineering Precursor Supply

Precursor supply is defined as the enhancement of the availability of primary metabolites or molecules derived from primary metabolism involved in the biosynthesis of natural products (Shiba et al., 2007). Precursor supply engineering can be achieved by manipulating either the pathways or enzymes involved with the precursor supply. Malonyl-CoA and methylmalonyl-CoA are the most commonly used and metabolically available precursors for the biosynthesis of polyketides. One study found that supplying methyl oleate enhanced the internal concentration of methylmalonyl-CoA, which is a biosynthetic precursor for FK506, and led to a 2.5-fold increase in FK506 production in *Streptomyces clavuligerus* CKD1119 (Mo et al., 2009). In another study, propionyl-CoA carboxylase with supplementation of propionate was found to effectively increase methylmalonyl-CoA and rapamycin titers in the mutant strain *S. rapamycinicus* UV_{2-2} induced by ultraviolet mutagenesis in comparison to wild-type strain (7 ∼ 8 mg/L) (Jung et al., 2011). The mutant strain was found to have a 3.2-fold improvement (23.6 mg/L) in comparison to wild-type strain *S. rapamycinicus* ATCC 29253 (7 ∼ 8 mg/L) (Jung et al., 2011). Further, Méndez and coworkers improved precursor metabolite pools for the production of the antitumor polyketide mithramycin in *Streptomyces argillaceus* by increasing the precursor supply of malonyl-CoA and glucose-1-phosphate (Zabala et al., 2013). Several classes of natural products utilize aromatic amino acids or other metabolites derived from the shikimate pathway as precursors, including flavonoids, alkaloids, polyketides, and non-ribosomal peptides (Knaggs, 2001). The production of the vancomycin analog balhimycin was increased 2.5-fold in *Amycolatopsis* sp. Y-89,21022. This was achieved by increasing the non-ribosomal peptide precursor 3-deoxy-D-arabino-heptulosonate7-phosphate synthase, the first enzyme in the shikimate pathway (Thykaer et al., 2010). In addition, manipulating key enzymes that direct carbon flux through core biochemical pathways involved in glucose, fatty acid, and amino acid metabolism can increase biosynthetic precursor pools. A study on the modulation of carbon flux between the pentose phosphate pathway and the glycolysis pathway found that a deletion of phosphofructokinase isoenzymes led to the enhanced production of antibiotics actinorhodin and undecylprodigiosin in *S. coelicolor* by increasing carbon flux through the pentose phosphate pathway (Borodina et al., 2008).

Precursor supply engineering has been successfully used to produce most of the major classes of natural products, with application to heterologous producing strains as well as native producers. When the native host is slow-growing or cannot be easily manipulated genetically, this process could be performed effectively in an appropriate heterologous host. A study bypassed the native deoxyxylulose 5-phosphate pathway and instead introduced the mevalonate pathway from *S. cerevisiae* to *E. coli*, which allowed for an increased production of amorpha-4,11-diene which is a precursor to antimalarial artemisinin (Martin et al., 2003). Combined approach of exogenous supplementation and engineering of intracellular pathway responsible for precursors can be also performed in a heterologous host. The biosynthetic process of the hybrid non-ribosomal peptide-polyketide yersiniabactin was known to rely on the supply of salicylate, L-cysteine, *S*-adenosyl-L-methionine, malonyl-CoA, and NADPH. When exogenous cysteine was fed to the culture of *E. coli* harboring yersiniabactin BGC and an additional set of genes (*hmwp1-2*) responsible for yersiniabactin precursor biosynthesis was introduced, the yersiniabactin production in *E. coli* was boosted to approximately 175 mg/L (Ahmadi and Pfeifer, 2016).

Precursor engineering strategy can be also employed to increase recombinant protein production by reducing unwanted by-products. One of the primary obstacles observed in high cell density cultivations of *E. coli* for the production of recombinant proteins is the formation of acetate, which is a by-product caused by an excess influx of carbon during aerobic fermentation. This acetate accumulation hampers cell growth and recombinant protein formation, even at low concentrations (Waegeman and Soetaert, 2011). A number of engineering approaches have focused on minimizing acetate formation in order to enhance recombinant protein production in *E. coli*. When a heterologous anaplerotic pyruvate carboxylase from *Rhizobium etli* is overexpressed in *E. coli*, the resulting strain had a 57% reduction in acetate formation and a 68% increase in β-galactosidase production (March et al., 2002). It is also possible to combine different strategies to reduce the formation of undesired by-products, including acetate. For example, one study found that a mutant *E. coli* strain containing a defective acetate pathway and an overexpressed phosphoenolpyruvate carboxylase-encoding *ppC* reduced acetate and other by-product formation and produced five time more β-galactosidase activity when compared the wild type strain (De Mey et al., 2010).

Pathway Engineering

Metabolic pathway engineering can be performed in the native host through repetitive gene expression, gene deletion, and introduction of new genes to enhance production of natural products (Pickens et al., 2011). For example, overexpression of the 4–12 tandem copies of the actinorhodin cluster resulted in a 20-fold increase in actinorhodin production in *S. coelicolor* (Murakami et al., 2011). Additionally, a *S. hygroscopicus* strain with 3–5 tandem copies of the 40 kb validomycin A cluster showed a 34% increase in production and a maximum titer of approximately 20 g/L (Zhou et al., 2014). Deletion of genes may be useful to eliminate competing pathways that may siphon off

important precursors or intermediates, or simply contribute to an unnecessary use of cellular resources which result to improve yields of products of interest. 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. The responsible hydrolase BioH was knocked out to improve simvastatin production (Xie et al., 2007). The regulatory component of the pathway can be manipulated to enhance production of the resulting natural product. Negative regulation by pathway specific repressors can help regulate secondary metabolite pathways. For example, one study improved the titer by 100-fold of antibiotics platensimycin (323 mg/L) and platencin (255 mg/L) through the inactivation of a gene encoding protein PtmR1 belonging to GntR family of transcriptional repressors (Smanski et al., 2009). On the other side of the spectrum, *Streptomyces* antibiotic regulatory protein (SARP) is a positive regulator of antibiotic production (Chen et al., 2010). Overexpression of SARPs and/or increasing SARP gene dosage using multi-copy plasmids has been demonstrated to increase production titers. Overexpression of *mgsA* or *chxA*, SARP family members that are positive regulators for the iso-migrastatin and cycloheximide biosynthetic machinery, respectively, in *Streptomyces amphibiosporus* ATCC 53964 led to a fivefold increased production of antibiotic lactimidomycin (Zhang et al., 2016). Members of the large ATP-binding regulators of the LuxR (LAL) family also generally function as transcriptional activators, and constitutive overexpression of these LAL-type activators was found to increase production of rapamycin in *S. rapamycinicus* and FK506 in *S. tsukubaensis* (Kušèer et al., 2007; Mo et al., 2012).

Competing pathways can also be deleted to ensure the production of important precursors or intermediates and to save useful cellular resources. When deleting pathways, the idea is to create a host with a minimized genome to ensure the efficient production of necessary secondary metabolites. Deleting non-essential genes and directing cellular resources toward pathways that are essential for the survival and product biosynthesis can improve cellular efficiency and streamline biochemical production. For example, the genome of *Streptomyces avermitilis* was effectively minimized to 83% of its original size. When heterologous streptomycin gene cluster was introduced into the genome-minimized *S. avermitilis*, the resulting strain produced a higher titer of streptomycin than both the parent *S. avermitilis* carrying the same heterologous gene cluster and the native streptomycin producer *S. griseus* (Komatsu et al., 2010). However, large scale deletions may result in unintended effects as the complete workings of the cell are not yet entirely understood.

Similar approaches have been employed to improve the secretion capability and productivity of biologics. Engineering the protein trafficking pathway represents one successful approach to improve the secretion of heterologous recombinant proteins. For example, the secretion of the heterologous proteins human insulin precursor and α-amylase from *A. oryzae* in *S. cerevisiae* was improved by the over-expression of Sec1/Munc18 proteins, which are involved in the protein secretory pathway (Hou et al., 2012). Increasing the copy number of genes that are associated with protein secretion can also enhance protein secretion. This is seen with the *Necator americanus* secretory protein (Na-ASP1), which shows potential as a vaccine protein for hookworm infections. Increasing the Na-ASP1 gene copy number caused saturation of secretory capacity in *P. pastoris*, a species of methylotrophic yeast, led to a decreased amount of secreted protein. This was remedied by the overexpression of the protein disulfide isomerase, which allowed for the increased secretion of Na-ASP1 protein in high copy clones (Inan et al., 2006). Another study showed that deletion of obstructive protease genes involved in fission could lead to the enhanced secretion of protease-sensitive human growth hormones (hGH) in *Schizosaccharomyces pombe*. The production of hGH was hampered by the intracellular retention of secretory hGH, and it was determined that the multi-protease deletant strain plays a role in hGH retention. Deletion of *vps10*, which encodes a carboxypeptidase Y sorting receptor and is involved in the traffic between the late-Golgi and prevacuolar compartments, resulted in an approximate twofold increase in hGH secretion (Idiris et al., 2006).

Combinatorial Biosynthesis

Combinatorial biosynthesis is one genetic engineering application that can modify biosynthetic pathways in order to yield new and altered natural product structures (Hopwood et al., 1985). This approach exploits indiscriminate substrates and uses engineered enzymes and pathways for the production of new natural product analogs.

Modular megasynthases, such as PKS and NRPS enzymes, constitute a class of multifunctional proteins that govern complex enzymatic mechanisms and catalyze multiple reactions useful for combinatorial biosynthesis. Type 1 PKSs consist of multiple modules which are responsible for incorporating acyl-CoAs into a polyketide backbone for elongation. Meanwhile, NRPSs are composed of a modular set of repeating enzyme domains for the activation and incorporation of amino acids (Park and Yoon, 2015). The modular NRPSs typically consist of a condensation domain, adenylation domain, and a thiolation domain, while type I PKSs generally contain a ketosynthase domain, acyltransferase domain, and an acyl carrier protein (Komaki et al., 2015; Skiba et al., 2018). Natural product structures can be modified by mixing and matching the megasynthases at the subunit, module, and domain levels. Genetic manipulation of PKS and NRPS encoding genes can result in predictable changes in structure that is difficult to achieve with standard chemical derivatization or total synthesis methods (Park et al., 2010). This approach to manipulating substrate incorporation and biosynthetic PKS and NRPS machinery has allowed for the generation of a great number of natural product analogs. Examples include erythromycin (McDaniel et al., 1999), pikromycin from type I modular PKS (Yoon et al., 2002) and daptomycin from NRPS (Robbel and Marahiel, 2010).

Post-assembly modifications, such as glycosylation, oxidation, and halogenation are performed by diverse enzymes and can lead to structurally and biologically diverse natural compounds (Park et al., 2010). Sugar moieties attached to the core structure of polyketides or non-ribosomal peptides by glycosyltransferases can also contribute to an extension of combinatorial biosynthesis.

Since several glycosyltransferases have been known to be flexible toward sugar donors and sugar accepters, arrays of analogs differing in glycosylation patterns via tailoring enzymes can also be generated by combinatorial engineering of glycosyltransferases from different pathways. For example, one study found that *A. orientalis*-derived glycosyltransferases accepted the unnatural sugar 4-*epi*-vancosamine in the presence of vancomycin pseudoaglycone or the glucosylated teicoplanin scaffold to generate novel hybrid glycopeptide compounds such as 4-*epi*-vancosaminyl form of vancomycin (Losey et al., 2001). Besides sugar biosynthesis, combinatorial biosynthesis can be applied for other modifications such as oxidation and halogenation. Oxidase genes from polyketide pathways have been used to induce structural alterations of important functional groups that are essential for biological activities. It has been reported that 5-*O*-desosaminyl erythronolide A, a potent precursor of ketolides and the latest generation of antibiotic compounds derived from erythromycin A, was produced by expressing the monooxidase gene *pikC* from the pikromycin pathway in a mutant strain of *S. erythraea* lacking of a EryBV glycosyltransferase (Basnet et al., 2008). In addition, a recent study obtained nine analogs of the antitumor antibiotic xantholipin through the individual in-frame mutagenesis of five tailoring enzymes (Zhang et al., 2012). In another study, fluorosalinosporamide, a derivative of the potent anticancer agent salinosporamide A, was produced by replacing the chlorinase gene *salL* from *Salinispora tropica* with the fluorinase gene *flA* from *Salinispora cattleya* (Eustaquio et al., 2010).

However, a common concern with this approach regards limited tolerance of downstream enzymes or domains to the new substance introduced by combinatorial biosynthesis and metabolic engineering. Rational design or directed evolution is one solution to this concern. Rational design is the strategy of creating new molecules with a certain functionality based on predicting how the molecule's structure will affect its behavior, while directed evolution refers to methods to alter enzyme function using mutagenesis and selection (Nannemann et al., 2011). In a recent study, the reactivity of PikC was modified through protein engineering driven by molecular dynamics and quantum mechanical calculations. The computation-driven PikC engineering yielded a $PikC_{D50N}$ mutant that showed improved catalytic efficiency compared to the wild-type PikC (Narayan et al., 2015). This study demonstrated that a rationally designed protein using a crystal structure of protein and/or a computational analysis can develop a predictive model for substrate scope and selectivity of natural product biosynthesis-mediated reactions. Directed evolution is also a powerful tool to modify the activity of key enzymes responsible for the biosynthesis of natural products and can lead a higher diversity of natural products by generating novel and more potent analogs (Williams et al., 2013). As an example, a few rounds of directed evolution restored and enhanced the activity of an impaired chimerical enterobactin NRPS that has been swapped with a non-cognate aryl-carrier protein (Zhou et al., 2007). In order to reduce the risk of limited tolerance and reduce concerns of efficiency, directed evolution requires a large, high-quality library and an efficient screening strategy. The swapping of functional domains often results in non-functional or heavily impaired chimerical enzymes, and this remains an existing problem when manipulating modular PKS and NRPS systems.

Mutasynthesis

Novel natural product analogs can also be generated through gene disruption and mutasynthesis. Disruption of a gene, such as a tailoring enzyme acting downstream in a pathway, can serve to introduce a structural change. Two FK506 analogs, 9-deoxo-31-*O*-demethylFK506 and 31-*O*-demethylFK506, were produced by targeting gene disruption in *Streptomyces* sp. MA6548 (Shafiee et al., 1997; Ban et al., 2013). These two recombinant mutants were genetically engineered via disruption of *fkbD* and *fkbM* genes that code for 31-*O*-demethylFK506 methyltransferase and 9-deoxo-31-demethylFK506 hydroxylase/oxidase (Shafiee et al., 1997; Ban et al., 2013). Inactivation of individual domains within the multidomain modular PKSs and NRPSs serves as an alternative to the deletion of a whole gene. Mutasynthesis involves the coupling of a gene inactivation strategy with precursor feeding to generate new structural analogs. Precursor feeding is useful due to the substrate-promiscuity of the biosynthetic enzyme. Precursor feeding may lead to the acceptance of similar substrates or mutasynthons, a natural substrate substitute that can replace the natural substrate of a disrupted gene after being added to the growth medium, to ultimately generate new analogs. Mutasynthesis can generate new analogs for many classes of compounds. For example, the analog cahuitamycin D was produced through mutasynthetic generation with twofold-enhanced biofilm inhibitory activity in comparison to its natural product counterpart (Park et al., 2016). Recently, this approach was applied to generate nonbenzoquinone analogs of the Hsp90 inhibitor geldanamycin, which has anti-proliferative activity on tumor cells (Shin et al., 2008; Wu et al., 2011). By removing the biosynthetic genes for the 3-amino-5-hydroxybenzoic acid starter unit and feeding the culture with various 3-aminobenzoic acids and related heterocycles, a chloro-substituted nonbenzoquinone analog with significantly improved therapeutic properties was produced along with other geldanamycin analogs (Kim et al., 2007, 2009). This has been also seen in the generation of new analogs of rapamycin (Khaw et al., 1998), balhimycin (Weist et al., 2002), and novobiocin/chlorobiocin (Li and Heide, 2005).

FUTURE PROSPECTS

An increasing number of natural products and natural product-derived compounds have been launched over the years (Butler et al., 2014). Since 2000, 77% of FDA-approved antibiotics are natural products, all of which were derived from microbes (Patridge et al., 2016). There have been extensive reviews of natural products, semi-synthetic natural products, and nature-inspired molecules currently approved by the FDA that show the continued importance of natural products for medicine and health (Sanchez et al., 2012; Newman and Cragg, 2016). Microbial

biologics are expected to remain prominent in the global biologics market, which was valued at 277 billion USD in 2015 and was recently estimated to reach 400 billion USD by 2025 (Grand View Research, 2017). While many of the biological activities of microbial natural products and biologics are well known, new advances and insights continue to be discovered. Chemical diversity from microbial natural products continue to be relevant to future drug discovery, with a continuing need for novel drugs with antibiotic, anticancer, and immunosuppressant effects, along with other pharmacological activities (Sanchez et al., 2012).

At the same time, there are a multitude of challenges facing microbial production of natural products and biologics. Some challenges to natural products-based drug discovery involve low production titers, difficulty in product isolation or structural identification. Similarly, there is much room for improvement in terms of the expression of recombinant proteins in microbial platforms. Accumulation of the end product in the microbial cell can cause global stress responses that result in cell growth inhibition. Also, the formation of misfolded and biologically inactive proteins can lower the yield of recombinant proteins. In particular, membrane proteins, high-molecular weight proteins, and multi-domain proteins are often expressed in inclusion bodies. Additionally, expressing eukaryotic proteins in a prokaryotic-based heterologous system can result in a product that is not correctly modified by post-translational enzymes, which are often required for functionality (Rosano and Ceccarelli, 2014). However, a wide variety of engineering strategies can be used with the conventional recombinant DNA technologies, including genome editing, ribosome engineering, precursor engineering, mutagenesis, and overexpression of structural genes, making it possible to facilitate the efficient production of natural products and pharmaceuticals in microbial systems.

Current technologies, such as CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein), should also be considered as tools for genome editing for additional improvements and to increase production (Gaj et al., 2013). For example, *in vitro* CRISPR-Cas9 cloning with Gibson assembly has provided an alternate strategy for heterologous expression of cryptic BGCs from genetically recalcitrant actinomycetes strains (Wang J.W. et al., 2015; Jiang and Zhu, 2016). Additionally, CRISPR-Cas9 has many prospects in the analyses of biochemical pathways in *Streptomyces* strains due to the development of a pCRISPomyces expression system (Cobb et al., 2015). With genome editing tools, it is also possible that non-model native hosts can be engineered to be heterologous production hosts to become platforms for combinatorial biosynthesis to create synthetic natural products and natural product derivatives. Some intrinsic limitations for non-model microbial hosts can be also improved by this genetic modification. *Corynebacterium glutamicum*, a GRAS organism, has been used for the industrial production of various amino acids for over five decades (Becker et al., 2011). Recently, it also showed promising potential for use as a protein expression system (Kallscheuer et al., 2016). However, this bacterium has intrinsic disadvantages, including a much lower transformation efficiency and lower yields of protein production. The CRISPR/Cas9 system was successfully employed to disrupt four different genes in *C. glutamicum*, opening new possibilities to use non-model strains as improved cell factories for the production of recombinant proteins and natural products (Peng et al., 2017).

Genome mining is another alternative process to discover secondary metabolites and is done by extracting information from genome sequencing (Bachmann et al., 2014; Zhongyue et al., 2018). Evaluating silent cryptic BGCs through genome mining has provided valuable avenues to generate novel molecules. For example, a genome mining strategy combined with bioinformatics predictions was used to isolate the novel natural product orfamide A by feeding a predicted precursor to a culture of *Pseudomonas fluorescens* (Zerikly and Challis, 2009). In a recent study, genome mining-based combinatorial biosynthesis approach also led to the discovery of new members of the leinamycin family of natural products. Leinamycin has been considered a promising anticancer drug lead due to its potent anticancer activities, unique molecular architecture and interesting modes of action. However, no leinamycin analog had been isolated in the past three decades until this study (Pan et al., 2017).

A combination of approaches can also lead to improvements in the field of microbial natural products, such as gene shuffling and ribosome engineering for increased secondary metabolite production. Additionally, the integration of 'omics' information has great potential in natural product drug discovery, such as with metabolomics to accurately quantify biochemical changes and metabolic pathways. Advancements in metagenomics has allowed for further understanding of diverse and complex microbial sources, including lakes, rivers, marine environments and extreme conditions, such as sub-seafloor sites and ice cores (Chandra Mohana et al., 2018).

In terms of structural characterization, X-ray crystallography and cryo-electron microscopy are advanced techniques that can allow for structure solving with high precision. Cryo-electron microscopy has been a leading method for evaluating macromolecular structures at near-atom resolution (Shoemaker and Ando, 2018). For example, single particle electron cryo-microscopy has been used to visualize pikromycin PKS module 5 from *S. venezuelae*, which allowed for 3D map construction with resolutions of 7.3–9.5 Å to reveal secondary structures (Dutta et al., 2014). These techniques are just a few among many that should be considered for structural studies on natural product biosynthesis. Advancements in computational strategies have led to the identification of BGCs in genome sequences and predictions of product chemical structures. Sequencing campaigns for natural product discovery should be directed toward samples likely to yield novel natural products along with well-characterized clades, such as actinomycetes, as they are still a resource for natural product discovery and have yet to be fully exhausted. The development of algorithms to mine the ever-increasing amounts of metagenomic data will allow for

the potential of genome mining to be realized (Medema and Fischbach, 2015). Finally, developing additional host platforms for high-throughput refactoring and functional expression of pathways has the potential to overcome current limitations in precursor supply, product toxicity, the ability to express very large gene clusters and more (Schmidt-Dannert, 2015).

Overall, microbial natural products and biologics will continue to broaden their diverse and integral role in human life. The potential for recombinant drugs is expanding through the utilization of new protein production platforms and efforts in product improvement. Microbial cells will remain as potent protein factories because of their versatility and cost-effectiveness. Engineering strategies and recombinant DNA technologies will also allow for the increased production of microbial natural products and recombinant proteins despite the many challenges faced. Continued efforts in natural product analog development will provide an avenue for the discovery of compounds with improved biological activities in comparison to their natural counterparts. Current advanced technologies can be utilized to further advance the field of microbial natural products, which remain a steadfast resource for novel compounds in drug discovery.

AUTHOR CONTRIBUTIONS

SP and YY designed, directed, and coordinated this project. JP, MY, AF, MM, NM, JW, EK, HC, JR, MCS, SP, and YY made substantial contributions in providing critical feedback and drafting the manuscript. SP and JP reviewed the final manuscript.

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