Leveraging synthetic biology for producing bioactive polyketides and non-ribosomal peptides in bacterial heterologous hosts

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Abstract

Bacteria have historically been a rich source of natural products (e.g. polyketides and non-ribosomal peptides) that possess medically-relevant activities. Despite extensive discovery programs in both industry and academia, a plethora of biosynthetic pathways remain uncharacterized and the corresponding molecular products untested for potential bioactivities. This knowledge gap comes in part from the fact that many putative natural product producers have not been cultured in conventional laboratory settings in which the corresponding products are produced at detectable levels. Next-generation sequencing technologies are further increasing the knowledge gap by obtaining metagenomic sequence information from complex communities where production of the desired compound cannot be isolated in the laboratory. For these reasons, many groups are turning to synthetic biology to produce putative natural products in heterologous hosts. This strategy depends on the ability to heterologously express putative biosynthetic gene clusters and produce relevant quantities of the corresponding products. Actinobacteria remain the most abundant source of natural products and the most promising heterologous hosts for natural product discovery and production. However, researchers are discovering more natural products from other groups of bacteria, such as myxobacteria and cyanobacteria. Therefore, phylogenetically similar heterologous hosts have become promising candidates for synthesizing these novel molecules. The downside of working with these microbes is the lack of well-characterized genetic tools for optimizing expression of gene clusters and product titers. This review examines heterologous expression of natural product gene clusters in terms of the motivations for this research, the traits desired in an ideal host, tools available to the field, and a survey of recent progress.

1. Introduction

Bacteria are valuable sources of natural products with medically relevant activities.[1] For most of the twentieth century, more than 80% of medical compounds were derived from or inspired by natural products.[2] Almost half of the natural products synthesized by bacteria possess some bioactivity, including antibiotic, anticancer, and immunosuppressant activities.[3] Demand for novel drugs with improved activities is increasing in part to the rise in multi-drug resistant infections and the ever-present need for a diverse set of cancer treatments.[4,5] Two of the most intriguing classes of natural products are polyketides and non-ribosomal peptides, which contribute considerably to the number of known bioactive natural products. These compound families are made by megaenzymes with multiple catalytic domains in an assembly-line fashion. Since their discovery, the modular nature of these enzymes has promised the ability to use combinatorial biosynthesis to produce diverse compounds that could be screened for novel bioactivity.[6] However, after the initial explosion of new antibiotics in the 1950's and 1960's, the role of natural products in drug development decreased considerably as synthetic chemistry techniques accelerated the pace of discovery beyond the speed with which new bioactive compounds could be isolated from novel microbes and/or their underlying biosynthetic machinery engineered to produce diverse compound libraries.[7] With the advent of next-generation DNA sequencing technologies, the number of putative biosynthetic gene clusters (BGCs) encoding PKSs and NRPSs has since increased exponentially to over 70,000 clusters, but the number of clusters associated with specific compounds remains under 1,000.[8] This gap is caused in part by challenges in culturing native producers in conditions that maximize biosynthesis of the desired compound.

It has been estimated that 99% of bacteria have not yet been cultivated in conventional laboratory media[9], and many of the remaining bacteria have slow growth rates, do not produce natural products in tested cultivation conditions, and/or are not genetically tractable.[10,11] Even in cases where the native host has been cultivated, substantial engineering may be required to produce relevant levels of putative secondary metabolites.[12,13] For these reasons, heterologous expression has become an essential tool in the genomic era of natural product discovery and development. While heterologous expression of natural product BGCs can provide substantial advantages, pitfalls are often encountered when developing and deploying heterologous hosts. Unfortunately, the common heterologous expression workhorse, *Escherichia coli*, has not proven to be a widely useful host for producing complex natural products including polyketides and non-ribosomal peptides. Therefore many groups have turned to non-model bacteria for heterologous production.

Optimizing a BGC for expression in a heterologous host is commonly described as "refactoring". Refactoring a pathway includes several strategies for distributing coding sequences into synthetic genetic circuits. These synthetic circuits contain a combination of promoters, RBSs, and terminators with known transcriptional and translational activities.[14] Coding sequences may also be codon optimized using *de novo* DNA synthesis so that their codon usage matches that of the heterologous host.[15] Characterized synthetic biology tools, which include genetic tools and methods for genetically modifying BGCs and hosts of interest, are necessary for researchers to refactor BGCs for optimal expression in heterologous hosts.

In this review, we discuss the role of heterologous expression in the discovery and engineered production of bioactive polyketides and non-ribosomal peptides from bacteria. We contextualize these recent advancements by identifying the various groups of bacteria that

produce these compounds and by comparing the heterologous hosts that researchers are using to express BGCs of interest. Our comparisons will focus primarily on what synthetic biology tools are available for individual hosts and to what extent are researchers taking advantage of available tools to modify heterologous hosts and/or BGCs for improved production.

2. Motivations for the heterologous expression of BGCs

Polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) can generate extraordinary chemical diversity by incorporating a wide variety of substrates in the initiation and elongation biosynthetic steps.[16–18] Additional tailoring catalytic domains, deviations from the canonical assembly-line enzymology, and hybrid PKS-NRPS enzymes further increase the complexity of these enzymes.[19,20] For more information on PKS and NRPS enzymology, Fischbach and Walsh provide an in-depth review on the relevant enzymatic domains and mechanisms.[21]

There is considerable interest in engineering these enzymes to alter substrate specificity, increasing their potential as a source of novel natural products.[22,23] However, these large enzymes pose several challenges to manipulating the synthesis of their products. Genetic manipulation of the BGCs is nontrivial because clusters often range from 20 to 100 kb in size (with some >100 kb), and contain repetitive DNA sequences due to the modular structure of their encoded megaenzymes.[24]

Many techniques designed for specifically cloning large BGCs encoding PKSs and NRPSs are available, allowing researchers to reliably transfer BGCs to heterologous hosts. Traditionally, researchers have cloned BGCs of interest by constructing libraries from genomic DNA and screening for the correct clone with PCR.[11] Targeted methods for capturing BGCs from genomic DNA are a more efficient option. These methods take advantage of restriction

enzymes or programmable nucleases that can cleave desired DNA segments from the chromosome *in vitro*[25,26] Researchers can then use homologous recombination and/or ligation cloning techniques to directly capture BGCs of interest in an expression vector.[27] RecET direct cloning and yeast transformation-associated recombination (TAR) cloning are commonly used to clone large BGCs in *E. coli* and *Saccharomyces cerevisiae*, respectively.[28,29] DNA assembly methods have also been developed for constructing large BGCs from multiple DNA fragments, which allows researchers to refactor BGCs of interest. Large DNA molecules can be assembled *in vitro* or *in vivo* using Golden Gate cloning, single-stranded annealing, and homologous recombination in yeast.[30–32]

PKS and NRPS activity is dependent on accessory proteins that are present in the host. PKSs and NRPSs are only functional in the presence of a compatible phosphopantetheinyl transferase (PPTase), which is responsible for incorporating an essential post-translational modification to carrier protein domains.[33] In addition, NRPS adenylation domains, which are responsible for activating amino acid substrates for incorporation into the peptide product, often require the presence of an MbtH-like protein (MLP) in order to be fully functional.[34] MbtH is a protein encoded in the BGC for synthesis of the mycobactin siderophore from *Mycobacterium tuberculosis* and forms the basis for the MLP superfamily.[35] The exact function of MLPs is still an active area of research, but researchers have observed that they bind to their cognate adenylation domains and improve their solubility and substrate affinity.[36,37] These auxiliary proteins can be located outside the gene clusters encoding putative NRPSs/PKSs thereby imparting a requirement to co-express native or promiscuous variants in heterologous hosts.

A major bottleneck to discovering novel natural products is the fact that the overwhelming majority of genetic material in environmental samples originates from strains that

have not been cultured in the laboratory.[38] In order to retrieve BGCs from these strains, researchers have turned to metagenomic libraries and/or commercial DNA synthesis of refactored BGCs prior to introducing them into a heterologous host (Figure 1).[15,39] Even at $0.10 per base pair, the cost of synthesizing large clusters is out of reach for most academic laboratories and a limitation to industrial efforts. Similarly, the cost of screening metagenomic libraries in search of clones harboring the desired cluster can be equally large and depend on either PCR, sequencing, or functional assays.[40] Functional screens include bacterial and/or fungal growth inhibition, detection of pigmented compounds, biosensor activation, and high-throughput analytical chemistry (e.g. HPLC).[41–43] Early screenings of metagenomic libraries were mostly random, but sequencing environmental DNA samples has allowed researchers to identify libraries with BGCs related to those of secondary metabolites of interest.[44] Heterologous hosts chosen to express BGCs from metagenomic libraries can influence which natural products and corresponding BGCs are discovered because some promoters from the library may be poorly expressed in various hosts.[45] However, researchers have found that introducing foreign transcriptional regulators to heterologous hosts can improve expression from these promoters and therefore increase natural product production.[46,47]

Another challenge associated with isolating bioactive natural products from native hosts that are difficult to cultivate is producing them in amounts sufficient for further characterization and ultimately clinical trials.[48,49] While many natural products can be synthesized chemically, their structural complexity requires the use of many chemical steps that result in poor overall yield.[50] Biological production can simplify this process by reducing multiple chemical reactions to a single bioreactor, if a suitable host can be developed. Even when native bacteria containing a BGC of interest can be cultivated, other issues can prevent production of secondary metabolites

at levels required for subsequent functional testing. Native hosts often grow slowly and have strict nutrient requirements, limiting the ability to scale up production from native hosts. Many clusters are also not expressed in normal laboratory conditions and the conditions required for activating the expression of many BGCs remain unknown.[51] Instead, researchers are using synthetic biology strategies for activating expression of these clusters in the native host if tools are available. That said, genetic tools are less likely to be available for species isolated from the environment, making transfer of the BGC into a heterologous host a necessary strategy.[52] A heterologous host provides the opportunity to use a production strain that has characterized genetic tools and is more suited for industrial-scale production.

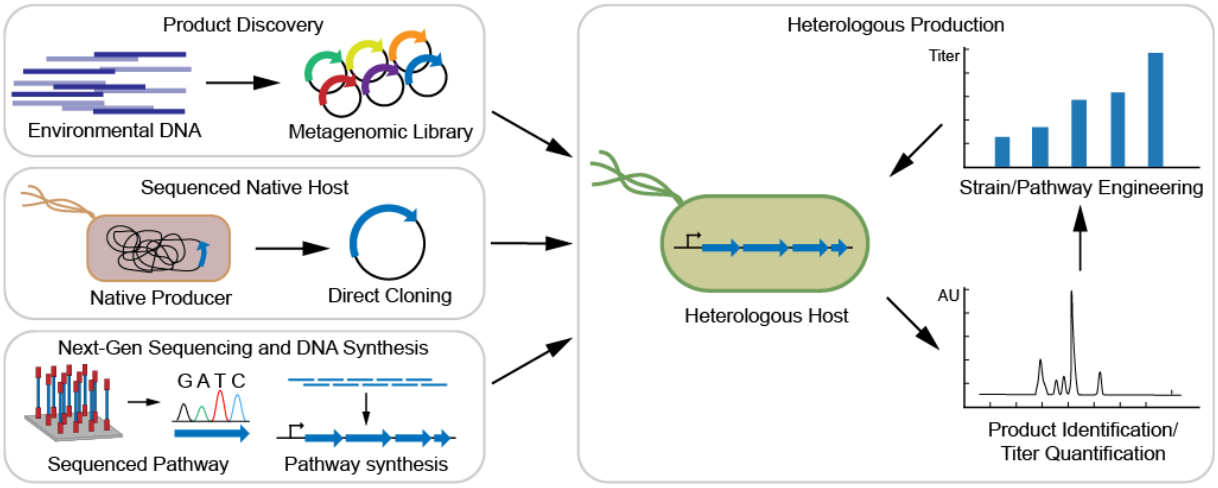

Figure 1. Strategies for discovering novel natural products using heterologous expression of BGCs.

Synthetic biology provides the necessary tools to manipulate BGCs and gain access to previously unknown natural products. Useful genetic tools and methods for expressing natural product clusters are characterized transformation protocols, promoters, ribosome binding sites (RBSs), terminators, replicative or integrative vectors for introducing heterologous genes, and methods for editing the genome.[53] To date, most advancements in bacterial synthetic biology have been applied to *E. coli*, but recently there has been a shift towards the development of genetic tools for non-model bacteria, including those from taxonomic groups known to synthesize polyketides and

non-ribosomal peptides.[54,55] Characterized promoters, RBSs, and terminators enable the predictable expression of heterologous BGCs. Methods for engineering the chromosome allow researchers to modify endogenous pathways. This strategy requires markerless and scarless methods that use a counterselection system. Earlier methods relied on counterselection genes that conferred sensitivity to a metabolite, such as *sacB* and sucrose or *upp* and 5-fluorouracil.[56,57] These methods require multiple steps to generate the desired strain and may require modifications to the genome *a priori* for the counterselection to be functional, so researchers have turned to counterselections based on CRISPR-associated nucleases.[58,59] Scarless and markerless genome editing, including CRISPR-based methods, enables the generation of multiple mutations to the chromosome, allowing researchers to optimize multiple metabolic pathways in heterologous hosts for improved polyketide and non-ribosomal peptide production.[60–62]3. Ideal traits of heterologous hosts for producing polyketides and non-ribosomal peptides

The ideal heterologous host for producing polyketides and non-ribosomal peptides should have a growth rate suitable for industrial fermentations, a characterized synthetic biology toolbox, and a sufficient supply of precursor metabolites (Figure 2). Important genomic features include GC content and codon usage similar to the native host, unless the BGC can be codon optimized for the heterologous host. A lack of native BGCs reduces competition for limited metabolite supplies and simplifies downstream purifications.[63] Alternatively, competing BGCs can be deleted from the chromosome using genome editing methods described in this review. Particularly in the case of antibiotics production, heterologous hosts should also be resistant to the product of interest and have the ability to secrete the product from the cell.[64] Bacteria that are capable of unicellular growth are more suited to growth in liquid cultures compared to mycelial

cultures that can increase the media viscosity and lower oxygen transfer rates.[65] Ideal hosts also have the metabolic flexibility to convert common feedstocks to the product of interest, in contrast to many native hosts that have atypical and expensive nutrient requirements.

Table 1 provides a comparison of some of these characteristics for species that have successfully produced heterologous polyketides and non-ribosomal peptides: *Streptomyces lividans*, *Streptomyces albus*, *Streptomyces venezuelae*, *E. coli, Myxococcus xanthus*, *Pseudomonas putida*, *Bacillus subtilis*, *Anabaena* sp. PCC 7120 (hereafter PCC 7120*)*, and *Synechocystis* sp. PCC 6803 (hereafter PCC 6803). These strains are representative of most of the species of bacteria that researchers have used for heterologous production of polyketides and non-ribosomal peptides.

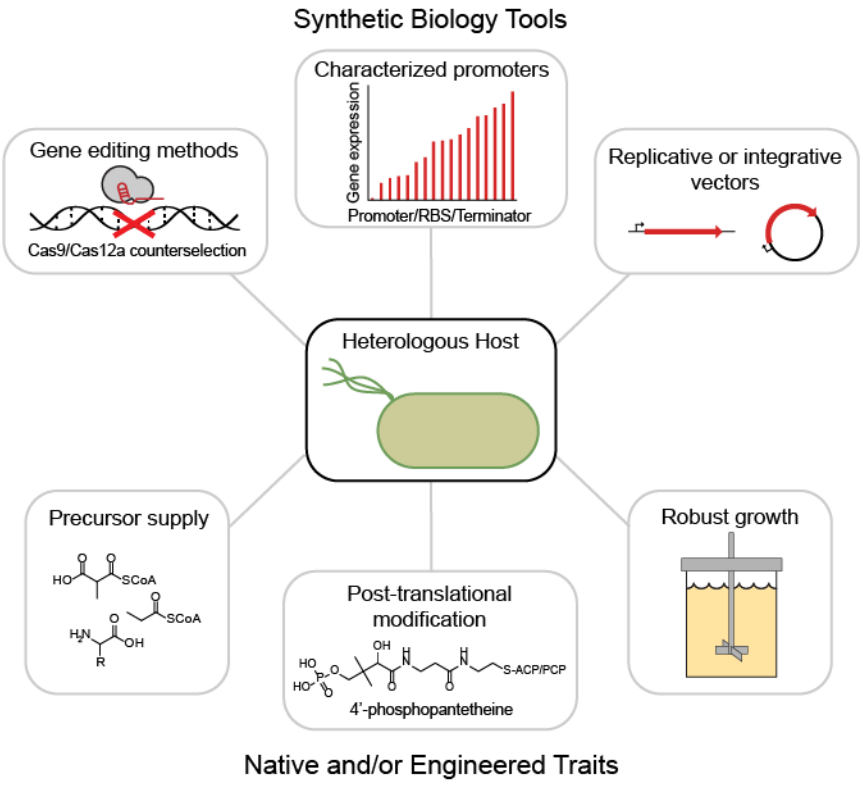

Figure 2. Synthetic biology tools and physiological traits required for production of polyketides and non-ribosomal peptides from a heterologous host.

Table 1. Comparison of phylogenetically diverse heterologous hosts for polyketide and non-ribosomal peptide production. –, not reported.

Strain	Phylum or Class	Genome Size (Mbp)	GC Content (%)	Doubling Time	Oxygen Requirement	Recombinant DNA Introduction (Max size)
S. lividans TK24	Actinobacteria	8.34	72.24	4.2 h	Obligate aerobe	Electroporation, conjugation (90 kb)[67]
S. albus J1074*	Actinobacteria	6.84	73.3	–	Obligate aerobe	Electroporation, conjugation (120 kb)[68]
S. venezuelae ATCC15439	Actinobacteria	9.05	71.7	1 h	Obligate aerobe	Electroporation (32 kb),[69] conjugation
E. coli BL21(DE3)	Gamma-proteobacteria	4.56	50.8	20 min	Facultative anaerobe	Chemical transformation, electroporation, conjugation (>100 kb)
P. putida KT2440	Gamma-proteobacteria	6.18	61.5	45 min	Obligate aerobe	Electroporation (30 kb), conjuation (60 kb)[70]
M. xanthus DK1622	Delta-proteobacteria	9.14	68.9	4-5 h	Obligate aerobe	Electroporation (60 kb)[70]
B. subtilis 168	Firmicutes	4.22	43.5	20 min	Facultative anaerobe	Electroporation, natural transformation
Anabaena sp. PCC 7120	Cyanobacteria	6.41	41.3	14 h	Photoautotroph	Conjugation (42 kb)[71]
Synechocystis sp. PCC 6803	Cyanobacteria	3.95	47.35	12 h	Photoautotroph	Conjugation

**S. albus* J1074 was recently reclassified as *Streptomyces albidoflavus*[66]

S. lividans and *S. albus* are two of the most common *Streptomyces* species used to study polyketides and non-ribosomal peptides from the natural product-rich actinobacteria. Compared to *E. coli* and *B. subtilis*, streptomycetes typically have slower growth rates and therefore low productivity rates for secondary metabolites.[72] *S. venezuelae* was recently identified as an alternative heterologous *Actinomyces* host because its doubling time is about half of that of *S. coelicolor*.[73,74] While not as plentiful as actinobacteria, a considerable number of bioactive natural products have been isolated from myxobacteria.[3] This bacterial class is also plagued with slow growth rates, with the model myxobacterium, *Myxococcus xanthus*, exhibiting a doubling time of 4-5 hours.[75]

P. putida has been proposed as a "hybrid" heterologous host for the production of polyketidess and non-ribosomal peptides. Its growth rate is similar to that of *E. coli*, it has a higher GC content similar to that of actinobacteria and myxobacteria, and pseudomonads encode many natural product BGCs in their genomes thereby providing native auxiliary proteins.[76,77] *B. subtilis* is another fast-growing heterologous host, and *Bacilli* species are a rich source of natural products.[78] The low GC content of *B. subtilis* makes it an attractive candidate for the

heterologous expression of BGCs from other low-GC bacteria and a difficult host for expressing the more common high GC content clusters from actinobacteria.

Cyanobacteria are an intriguing alternative to heterotrophic microorganisms for bioprocesses because they can convert sunlight and CO_2 into chemicals of interest.[79] PCC 7210 and PCC 6803 are both attractive cyanobacterial heterologous hosts for natural products and have similar doubling times of 14 and 12 hours, respectively.[80] Alternative cyanobacterial hosts have faster growth rates, including *Synechococcus elongatus* sp. PCC 7942, which has a doubling time of 5 hours, and a related strain, *S. elongatus* sp. UTEX 2973, grows twice as fast.[81] Heterologous production of a polyketide or non-ribosomal peptide has not yet been reported in UTEX 2973 or another fast-growing model species *Synnechococcus* sp. PCC 7002, which contains one native PKS.[82]

Researchers have characterized promoters for most of the heterologous hosts mentioned in Table 1, including *Streptomyces* species,[74,83] *P.putida*,[84,85] *B. subtilis*,[86] PCC 7120,[87] PCC 6803,[88] and of course, *E. coli.*[89] Most myxobacterial genetic tools have been developed for *M. xanthus*, and heterologous expression in this species has involved several constitutive promoters, but a promoter library or a reliable inducible promoter has not been reported in the literature.[90] Therefore, *M. xanthus* is limited in transcriptional control of heterologous genes compared to other candidate hosts.

Methods for integrating BGCs into the chromosome of heterologous hosts include transposition, phage integration, and homologous recombination. All three of these tools are available for *P. putida* and have been demonstrated for the chromosomal integration of BGCs.[84,91,92] Phage integration is a convenient method for introducing heterologous genes to *Streptomyces* because most species contain at least one phage integration site.[74] Transposition is

the most reliable method for stably integrating large (>50 kb) BGCs into the chromosome of *M. xanthus*, whereas phage integration cannot be used for constructs of this size.[70,93] RecA-mediated recombination is an efficient method for integrating exogenous DNA into the chromosome of *B. subtilis*.[94] Even though there are few examples of cyanobacteria being used as a heterologous host for BGCs, genetic tools have been developed for the heterologous production of natural products in these species.[95–98] Lastly, CRISPR-based genome editing methods are available for all of the heterologous hosts discussed in this review.[60,61,99–104]

Common substrates for PKSs and NRPSs include primary metabolites, such as acetyl-CoA, malonyl-CoA, and proteinogenic amino acids. However, many BGCs require substrates that are either not intrinsically synthesized or are synthesized in small quantities by some heterologous hosts.[16,17,105] This issue can universally be addressed by expressing a promiscuous coenzyme A ligase in the heterologous host and supplying the necessary substrate in the growth media.[106,107] There has also been success in engineering the *de novo* synthesis of metabolites, such as methylmalonyl-CoA, ethylmalonyl-CoA, and non-proteinogenic amino acids, by expressing heterologous pathways or by overexpressing native pathways.[108–110] These strategies have also been used to heterologously synthesize natural products functionalized with azide and alkyne groups, enabling semi-synthetic strategies for producing natural product derivatives that can be further diversified chemically.[111,112]

As mentioned previously, PPTases are essential for PKS and NRPS activity. PPTase genes are often located in BGCs encoding PKSs and NRPSs, but in the case that they are not, heterologous hosts must express a broad-range PPTase in order for the heterologous PKSs and NRPSs to be active.[33] PPTases are commonly found in actinobacteria,[113] and *E. coli* is the only heterologous host mentioned above that does not encode a PPTase in its genome. Pfeifer *et al*.

solved this issue by introducing *sfp*, a gene encoding a broad-range PPTase from *B. subtilis*.[114,115] Multiple cyanobacteria express genes encoding PPTases, and the PPTase from PCC 7120 appears to possess broad-range activity, but the one from PCC 6803 does not.[116,117] Both *M. xanthus* and *P. putida* have at least one *sfp*-type PPTase, and the one from *P. putida* has demonstrated a broad substrate range.[33,118,119] Unlike PPTases, MLPs from phylogenetically distant hosts are not interchangeable.[120,121] Often the MLP is encoded in the same BGC as the NRPS, but a considerable amount of BGCs do not encode an MLP, so care must be taken to provide a compatible MLP if they are to be transferred to a heterologous host.[122]

4. Recent examples of heterologous production of bioactive polyketides and non-ribosomal peptides

Beginning in 2013, there has been a consistent increase in the number of studies using bacterial heterologous expression to study or engineer the biosynthesis of polyketides and non-ribosomal peptides (Figure 3a). Unsurprisingly, actinobacteria are the most abundant source of sequenced BGCs encoding PKSs and NRPSs, and *Streptomyces* species have been the most common hosts for the heterologous expression of these BGCs (Figure 3b, 3c). They are also the most common heterologous hosts for the discovery of novel polyketides and non-ribosomal peptides through the heterologous expression of BGCs from metagenomic libraries and cryptic BGCs (Figure 3d). *E. coli* is the second most popular heterologous host, and it is most commonly engineered for improved production of polyketides and non-ribosomal peptides natively produced by non-proteobacteria, showing that researchers are taking advantage of the state-of-the-art synthetic biology tools available for *E. coli*. Studies using myxobacteria, pseudomonads, and *B. subtilis* as the heterologous host are less common, and in contrast to those using

Streptomyces, most are attempting to engineer the production of the polyketides and/or non-ribosomal peptides of interest.

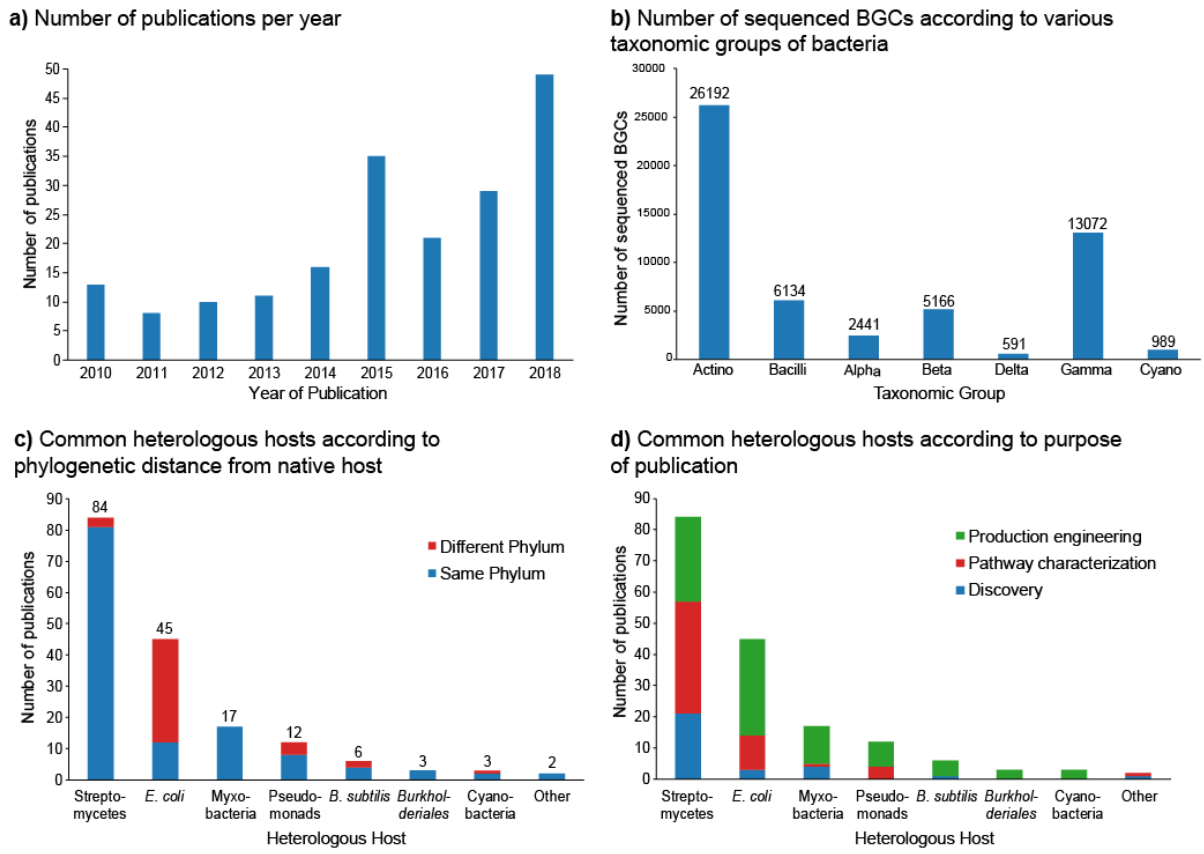

Figure 3. Trends and statistics impacting heterologous expression of BGCs in bacteria. A) Number of publications reporting heterologous expression of polyketide and non-ribosomal peptide BGCs per year. B) Number of sequenced BGCs encoding PKSs and/or NRPSs in the AntiSmash database. Data labels represent value on bar chart. All groups represent a bacterial class, except for the phylum cyanobacteria. Values were determined by building a query on the AntiSmash Database searching for clusters of the types "nrps" and/or "pks" for each taxonomic group of interest. The AntiSmash Database was accessed on January 6, 2019. C) Number of publications reporting heterologous expression of BGCs according to heterologous host and phylogenetic distance between the native host and heterologous host. Data labels represent total number of publications. D) Number of publications reporting heterologous expression of BGCs according to heterologous host and publication purpose. Data in panels C and D are limited to articles published from 2013 to 2019. Publications were identified via advanced searches in PubMed and Web of Science and supplemented by a manual literature review. The PubMed query was "(((heterologous[Title/Abstract] AND (expression[Title/Abstract] OR production)[Title/Abstract])) AND (polyketide OR nonribosomal peptide OR non-ribosomal peptide)) NOT (fungi[Title/Abstract] OR fungal[Title/Abstract] OR plant[Title/Abstract])". The Web of Science query was "ALL=(heterologous AND (expression OR production)) AND ALL=(polyketide OR nonribosomal peptide OR non-ribosomal peptide) NOT TS=(fungal OR fungi OR plant)". Irrelevant publications were manually removed from the search results.

Except for *E. coli*, heterologous hosts are most often used to express BGCs from strains of the same phylum (Figure 3c). Related strains are more likely to share characteristics important

for heterologous gene expression, such as codon usage and GC content.[123] This phylogenetic dependence is a limiting factor for the study of BGCs from cyanobacteria. Even though the number of bioactive secondary metabolites isolated from cyanobacteria has increased in recent years,[3] utilizing cyanobacteria for the heterologous production of polyketides and non-ribosomal peptides is still rare, and optimal heterologous hosts for the production of cyanobacterial secondary metabolites are still to be determined. There is a relatively low number of sequenced genomes and BGCs from myxobacteria and cyanobacteria compared to other taxonomic groups (Figure 3b), hindering the identification and heterologous expression of new BGCs from these groups.[124,125]

In the past two years, three publications have reported the capabilities of *Burkholderiales* sp. DSM 7029 (originally classified as *Polyangium brachysporum*, hereafter DSM 7029) as an alternative heterologous host for the production of myxobacterial natural products.[126–128] However, yields were 1-2 orders of magnitude lower compared to heterologous product yields from *M. xanthus*. Currently, *M. xanthus* remains the ideal heterologous host for producing myxobacterial natural products, but DSM 7029 could be developed for heterologously expressing BGCs from other betaproteobacteria, another natural product-rich class of bacteria.[129]

In the following sections, we discuss individual studies that report the heterologous production of nine bioactive polyketides and non-ribosomal peptides from the aforementioned heterologous hosts. Throughout our discussion, we highlight the synthetic biology techniques and metabolic engineering strategies mentioned in Section 3 that researchers have used to improve heterologous production. The structures of these products are depicted in Figure 4 and their properties are summarized in Table 2.

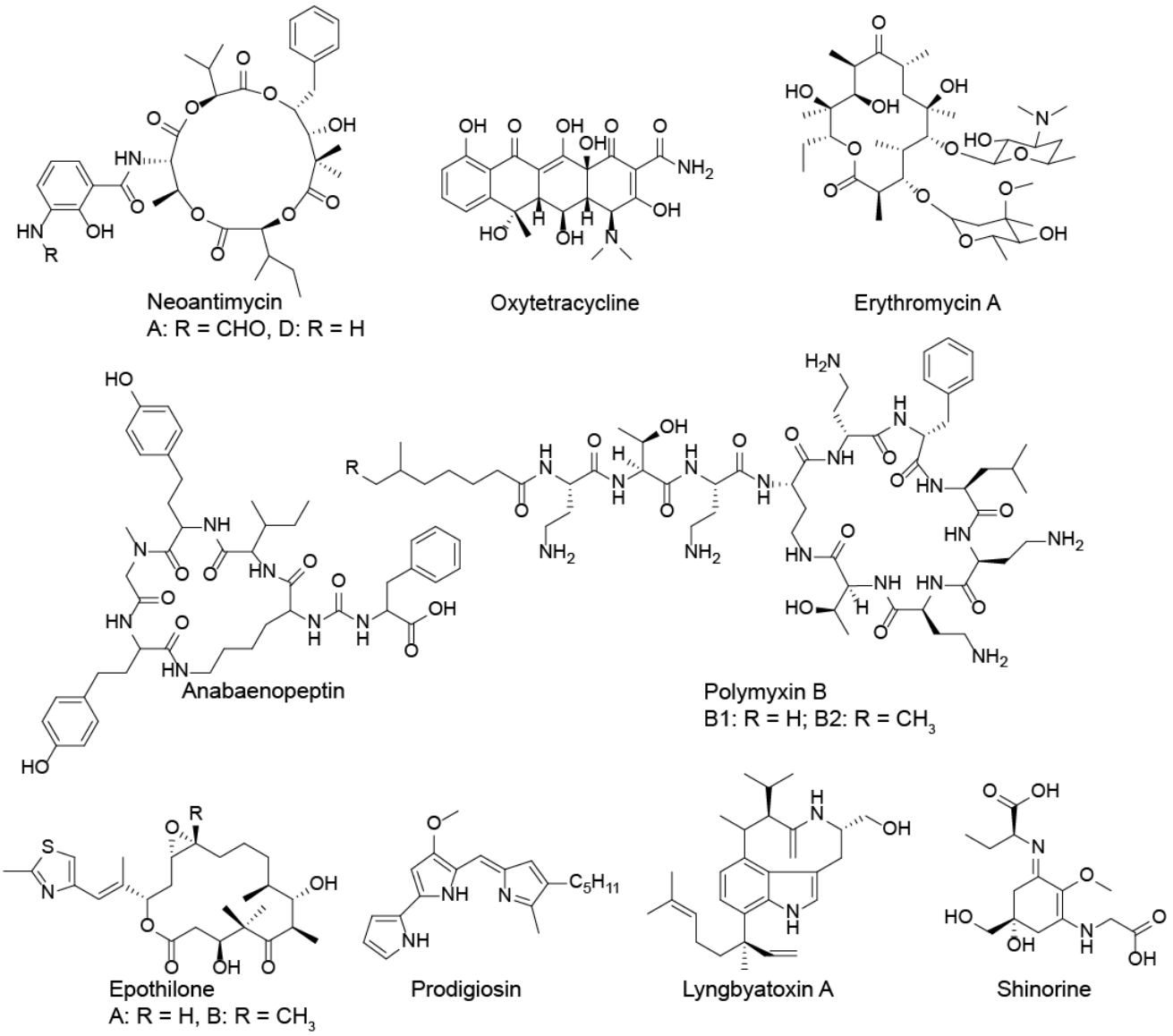

Figure 4. Structures of select bioactive polyketides and non-ribosomal peptides produced by heterologous hosts

4.1 Heterologous production in Streptomycetes

Actinobacteria have been the source for several prominent antibacterial compounds, such as daptomycin and chloramphenicol. Roughly 35% of all known microbial natural products with antinfective, antitumor, and antiviral activities were discovered from actinobacteria.[3] The development of model *Streptomyces* species as heterologous hosts will be essential for the discovery and production of actinobacterial natural products, as they will likely continue to be the major source of novel bacterial secondary metabolites. Recently, researchers have used

genetically tractable *Streptomyces* species for the heterologous production of the actinobacterial natural products, neoantimycins and oxytetracycline. Neoantimycins are hybrid polyketides/non-ribosomal peptides produced by several actinobacteria, and several derivatives have been found to induce apoptosis in cancer cells.[130,131] Neoantimycin A specifically inhibits the K-Ras GTPase that is implicated in the development of most pancreatic, colorectal, and lung cancers.[132] Oxytetracycline is a precursor for the semi-synthesis of tetracycline-based antibiotics.[133] Tetracycline and its derivatives interfere with the association of aminoacyl-tRNAs with the ribosome in bacteria thereby inhibiting protein synthesis.[134]

The BGCs producing neoantimycins from *Streptomyces conglobatus* and *Streptomyces orinoci* were recently characterized through heterologous expression of the clusters in *S. albus*.[135,136] Heterologous expression of the BGC from *S. conglobatus* yielded a novel derivative of neoantimycin that was up to 10 times more active toward cancer cell lines than cisplatin, a common anti-cancer drug. Cas9-assisted genome editing was used to introduce part of the neoantimycin BGC from *S. orinoci* into a related antimycin BGC on the chromosome of *S. albus* to generate a chimeric pathway that produces several neoantimycin derivatives at levels comparable to *S. orinoci*.

The fast growth rate of *S. venezuelae* makes it an attractive host for the heterologous production of actinobacterial polyketides and non-ribosomal peptides. Yin *et al.* successfully engineered *S. venezuelae* to heterologously produce oxytetracycline. Initially, the genes in the oxytetracycline BGC did not express in *S. venezuelae*, resulting in no oxytetracycline production. The authors improved transcription of the heterologous pathway by introducing a transcriptional activator from the native producer. They also increased intracellular levels of malonyl-CoA by overexpressing an acetyl-CoA carboxylase from *S. coelicolor*. The final oxytetracycline titer of

430 mg/L is comparable to that of the native host, *Streptomyces rimosus*, but *S. venezuelae* can achieve this titer in two days, while the native host takes eight, resulting in a four-fold increase in productivity through heterologous production.

4.2 Heterologous production in *E. coli*

The advanced synthetic biology tools characterized in *E. coli* and the wealth of knowledge of *E. coli* metabolism has allowed researchers to engineer *E. coli* for the heterologous production of a select few natural products. Recent compounds include the actinobacterial antibiotic, erythromycin, and anabaenopeptins, which have been discovered from multiple cyanobacteria.[137,138] Erythromycin inhibits protein synthesis in bacteria by binding to the 50S subunit of the ribosome and preventing translocation along the RNA transcript, and this antibiotic is still used today to treat bacterial infections.[139] The cyanobacterial natural products, anabaenopeptins, exhibit protease inhibitor activity, specifically against protein phosphatase I, an enzyme that regulates transcription of HIV-1.[138,140,141]

Erythromycin is one of the most studied actinobacterial polyketides, partly because its synthase is the first PKS to be functionally expressed in *E. coli*.[114] The entire BGC for erythromycin synthesis from *Saccharopolyspora erythrea* was first successfully reconstituted in *E. coli* by Zhang *et al.*, achieving titers up to 10 mg/L.[142] The availability of advanced synthetic biology tools and metabolic knowledge for *E. coli* has led to gradual increases in titers. Strategies for increasing erythromycin titers include engineering native metabolic pathways to increase intracellular concentrations of propionyl-CoA, introducing heterologous enzymes from *S. coelicolor* for methylmalonyl-CoA biosynthesis, and optimizing the expression plasmids encoding the erythromycin BGC.[143,144] The most recent study achieved an erythromycin titer of 40 mg/L.[145]

A recently developed strategy for cloning BGCs into heterologous hosts resulted in the heterologous production of cyanobacterial non-ribosomal peptides in *E. coli*.[146] Greunke *et al*. used Direct Pathway Cloning (DiPAC) to refactor the BGC for anabaenopeptin with *E. coli*-specific promoters and introduced the optimized pathway into *E. coli* via two replicative plasmids. The authors did not report an exact value for anabaenopeptin production, but HPLC/MS analysis revealed that their engineered strain of *E. coli* produced over 100 times more anabaenopeptins in 48 hours than what the native producer produced in 50 days.

These two studies demonstrate how well-characterized genetic tools can facilitate the heterologous expression of BGCs from phylogenetically distant bacteria. The favorable growth characteristics also make *E. coli* an attractive host for producing cyanobacterial natural products because it can reach higher cell densities and production titers in a much shorter time compared to cyanobacteria. However, some studies suggest that *E. coli* cannot be a widely useful heterologous host for natural products due to apparent toxicity from the expression of heterologous BGCs[28,147].

4.3 Heterologous production in *M. xanthus*

Of the heterologous hosts discussed in this review, *M. xanthus* appears to have the least developed synthetic biology toolbox available. However, several researchers have been able to improve the production of myxobacterial compounds in this heterologous host by optimizing transcription of the corresponding BGC. The antitumor compounds, epothilones have been a common target for heterologous production in *M. xanthus*. Epothilones bind to the β-tubulin subunit of microtubules, leading to their polymerization and inducing apoptosis in cancer cells.[148]

Heterologous production of epothilones in *M. xanthus* was first reported by Julien and Shah with titers approaching 0.5 mg/L.[149] Since then, several studies have attempted to improve the production of epothilone derivatives. Yue *et al*. discovered that several native promoters from *M. xanthus* are transcriptionally active in different stages of growth, resulting in low epothilone titers.[150] They constructed several tandem-repeat promoter variants to drive the expression of the epothilone BGC and improved epothilone titers two-fold. Peng *et al*. achieved similar results using CRISPR/dCas9 activation of the epothilone BGC. They fused the ω transcription factor to a catalytically inactive Cas9 (dCas9) and screened several sgRNA targets for improved epothilone production, demonstrating the advantages of transcriptional control in the heterologous production of natural products.

A recent study details an impressive attempt to completely refactor the epothilone BGC from *S. cellulosum* for heterologous expression in *M. xanthus*.[151] The authors codon optimized individual genes for expression in *M. xanthus* and distributed them into four operons using an established promoter, RBS, and terminator commonly used in *M. xanthus*. While epothilone production using the artificial pathway was much lower than other studies using the native pathway (100 μg/L vs. 20 mg/L), this effort shows a great step forward towards refactoring complex natural product BGCs in *M. xanthus*. Refactoring pathways into synthetic genetic circuits often requires testing multiple designs and can reduce production compared to the native pathway.[14] Future iterations of the artificial epothilone pathway should lead to improvements in heterologous production.

4.4 Heterologous production in *P. putida*

P. putida has been a popular choice for the heterologous production of polyketides and non-ribosomal peptides from proteobacteria, particularly those from myxobacteria. One early

study demonstrated that *P. putida* was capable of producing myxochromide S with titers up to 5 times higher than the native myxobacterial producer, *Stigmatella aurantiaca*.[152] However, more recent studies have reported low titers of heterologous products compared to alternative hosts.[70,90,153] Despite the limited success of *P. putida* as a heterologous host for natural products, many genetic tools have been developed for it within the last few years, so heterologous production from this species could likely be improved if researchers take advantage of the latest tools.

An exceptional study reports the heterologous production of prodigiosin, a pigmented polyketide/non-ribosomal peptide natively synthesized by *Serratia marcescens*.[154] Prodigiosin is an antibiotic that inhibits transcription in *E. coli*, but not *P. putida*.[64,155] Recently, there has been increased interest in prodigiosin due to its reported anticancer and immunosuppressant activities. It has displayed apoptotic and antimetastatic affects against multiple cancer cell lines,[156,157] and it is a T cell-specific immunosuppressant that inhibited the development of immune disease in mouse models.[158]

Domröse *et al*. introduced the BGC for prodigiosin synthesis through Tn5 transposition. The authors took advantage of the deep red color generated by prodigiosin and constructed a transposable vector with a promoterless copy of the BGC. After screening for transposon mutants that appeared as red colonies, they identified several mutants that had integrated the gene cluster downstream of a strong chromosomal promoter, resulting in prodigiosin titers of up to 150 mg/L.[159] This strategy enabled the discovery of strong native promoters for the heterologous expression of BGCs and improved prodigiosin production compared to a previous study that used a synthetic promoter.[160]

4.5 Heterologous production in *B. subtilis*

Despite the advantageous growth characteristics of *B. subtilis*, it is limited as a heterologous host for polyketides and non-ribosomal peptides because of the low GC content of its chromosome. For example, a refactored pathway for the synthesis of 6-deoxyerythronolide B, the precursor to erythromycin, in *B. subtilis* resulted in a titer of 2.6 μg/L,[161] much lower than what is possible in *E. coli*.[114] Several natural products have been discovered from low-GC bacteria, including other *Bacilli* and some cyanobacteria. One example of these compounds is a class of antibiotics called polymyxins. These compounds are bactericidal towards gram-negative bacteria, and their primary mode of action is increasing cell wall permeability.[162,163]

Kim *et al.* demonstrated the utility of *B. subtilis* for heterologously producing non-ribosomal peptides from other low-GC Bacilli by engineering a strain of *B. subtilis* to produce polymyxins, antibiotics natively produced by *Paenibacillus polymyxa*.[164] Integrating the polymyxin BGC into the chromosome of *B. subtilis* yielded a strain capable of producing up to 200 mg/L of polymyxins. The authors also succeeded in engineering strains towards the selective production of polymyxins B and E by replacing domains in the polymyxin NRPS with homologous domains with the desired substrate specificity.

4.6 Heterologous production in cyanobacteria

The development of cyanobacteria for the heterologous production of polyketides and non-ribosomal peptides is still in its infancy. However, researchers are poised to make rapid improvements in this field with the availability of well-characterized synthetic biology tools for cyanobacteria.[165] Roulet *et al.* have developed genetic tools specifically for polyketide production in *S. elongatus* and applied them to the heterologous production of multimethyl-branched esters.[95] Two exceptional studies, described below, demonstrate the capabilities of PCC 7120 and PCC 6803 for producing two bioactive non-ribosomal peptides, lyngbyatoxin A and

shinorine, respectively. Lyngbyatoxin A is a cytotoxin and acts as a tumor promoter in mammalian cells by activating protein kinase C, an enzyme that regulates cell proliferation and differentiation.[166] Shinorine is a UV-protective natural product that also exhibits antioxidant activity by activating the Keap1-Nrf2 pathway that is responsible for regulating the production of antioxidant enzymes in human cells and is therefore a common target for treating diseases caused by oxidative stress.[167]

PCC 7120 was used as a heterologous host for the production of lyngbyatoxin A, a cytotoxin naturally produced by *Moorea producens*.[71] The authors introduced the BGC for lyngbyatoxin A into PCC 7120 through a replicative vector. Initially, PCC 7120 was able to express the BGC using the native promoter from *M. producens* and produce lyngbyatoxin A, but the authors improved production by altering the nitrogen source and replacing the native promoter with promoters characterized for the heterologous host. Lyngbyatoxin A production was maximized and increased 13-fold to 3.2 mg/L when the strain was grown on nitrate and a strong constitutive promoter was used to drive expression of the BGC.

Yang *et al.* describe the only study so far on the heterologous production of a non-ribosomal peptide-based natural product in PCC 6803.[168] The authors introduced a BGC from *Fischerella* sp. PCC 9339 responsible for synthesizing shinorine using a replicative vector that co-expressed the shinorine BGC and the broad-range PPTase from PCC 7120.[169] Initially, imbalanced expression levels of individual genes in the BGC led to the accumulation of pathway intermediates and limited shinorine production. The authors removed this imbalance and improved production by inserting strong constitutive promoters upstream of the poorly expressed genes in the BGC, resulting in a titer of 0.71 mg/L of shinorine.

Remarkably, both heterologous hosts were able to generate natural product titers comparable to those of the native producers. The use of well-characterized promoters in these two studies demonstrates the importance of transcriptional control when expressing heterologous BGCs.

Table 2. Bioactive polyketides (PK) and non-ribosomal peptides (NRP) produced through heterologous expression in bacteria in recent studies.

Heterologous Host	Molecule	Native Host	Class	Bioactivity	Titer
S. albus	Neoantimycin	*Streptomyces conglobatus*	PK/NRP	Anticancer	n. d.[135,136]
S. venezuelae	Oxytetracycline	*Streptomyces rimosus*	PK	Antibiotic	430 mg/L[133]
E.coli	Erythromycin	*Saccharopolyspora erythrea*	PK	Antibiotic	40 mg/L[145]
	Anabaenopeptin	*Nostoc punctiforme*	NRP	Protease inhibitor	n. d.[146]
M. xanthus	Epothilone	*Sorangium cellulosum*	PK	Anticancer	21 mg/L[170]
P. putida	Prodigiosin	*Serratia marcescens*	PK/NRP	Antibiotic, Antitumor, Immunosuppressive	150 mg/L[159]
B. subtilis	Polymyxin	*Paenibacillus polymyxa*	NRP	Antibiotic	~200 mg/L[164]
Anabaena sp. PCC 7120	Lyngbyatoxin A	*Moorea producens*	NRP	Cytotoxin	3.2 mg/L[71]
Synechocystis sp. PCC 6803	Shinorine	*Fischerella* sp. PCC 9339	NRP	Antioxidant, sunscreen	0.71 mg/L[168]

5. Conclusions and Future Directions

Researchers have put considerable effort towards identifying alternative heterologous hosts for the discovery and production of polyketides and non-ribosomal peptides. *Streptomyces* species remain the dominant candidates, and this trend will hold in the foreseeable future as actinobacteria continue to be rich sources for bioactive natural products. Currently, heterologous hosts are most successful when expressing BGCs from phylogenetically similar species. However, for non-model heterologous hosts that are related to natural product-rich species, synthetic biology tools are limited and it is considerably more difficult to engineer these hosts for improved heterologous production. The development of new synthetic biology tools needs to

continue for these bacteria in order for heterologous hosts to become a more viable option for producing polyketides and non-ribosomal peptides. However, the model heterologous hosts for some bacterial taxa, such as myxobacteria and cyanobacteria, have slower growth rates and are more difficult to handle compared to other heterologous hosts.

Refactoring bacterial pathways in heterologous hosts has become a common strategy for pathways that do not produce polyketides and non-ribosomal peptides,[14,171] but when researchers do heterologously express PKSs and NRPSs, the native BGC is often left fully intact, requiring the use of potentially nonoptimal promoters, RBSs, and terminators. Attempts to refactor these BGCs are usually limited to introducing characterized promoters for 1-2 operons. This minimal approach can be improved upon by taking advantage of tools for designing RBSs and investigating effects of termination on gene expression in heterologous hosts.[172,173]

While genetic tools remain poorly characterized for many natural product-rich bacteria, phylogenetically distant species with better developed tools could become ideal hosts, provided heterologous BGCs are refactored for optimal expression. Successful attempts to refactor BGCs from actinobacteria and cyanobacteria for heterologous expression in *E. coli* demonstrates the utility in this strategy and suggests that the ideal heterologous host for producing polyketides and non-ribosomal peptides can be an unrelated species.[31,174] However, issues that synthetic biology cannot immediately address, such as observed toxic effects from expressing heterologous pathways in *E. coli*,[28,147] require that alternative hosts are developed for these projects. Improved synthetic biology tools will be necessary for non-model organisms if pathway refactoring is to be fully realized as a strategy for improving the heterologous production of polyketides and non-ribosomal peptides in these hosts.

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