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Bacillus subtilis as a host for natural product discovery and engineering of biosynthetic gene clusters

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Many bioactive natural products are synthesized by microorganisms that are either difficult or impossible to cultivate under laboratory conditions, or that produce only small amounts of the desired compound. By transferring biosynthetic gene clusters (BGCs) into alternative host organisms that are more easily cultured and engineered, larger quantities can be obtained and new analogues with potentially improved biological activity or other desirable properties can be generated. Moreover, expression of cryptic BGCs in a suitable host can facilitate the identification and characterization of novel natural products. Heterologous expression therefore represents a valuable tool for natural product discovery and engineering as it allows the study and manipulation of their biosynthetic pathways in a controlled setting, enabling innovative applications. *Bacillus* is a genus of Gram-positive bacteria that is widely used in industrial biotechnology as a host for the production of proteins from diverse origins, including enzymes and vaccines. However, despite numerous successful examples, *Bacillus* species remain underexploited as heterologous hosts for the expression of natural product BGCs. Here, we review important advantages that *Bacillus* species offer as expression hosts, such as high secretion capacity, natural competence for DNA uptake, and the increasing availability of a wide range of genetic tools for gene expression and strain engineering. We evaluate different strain optimization strategies and other critical factors that have improved the success and efficiency of heterologous natural product biosynthesis in *B. subtilis*. Finally, future perspectives for using *B. subtilis* as a heterologous host are discussed, identifying research gaps and promising areas that require further exploration.

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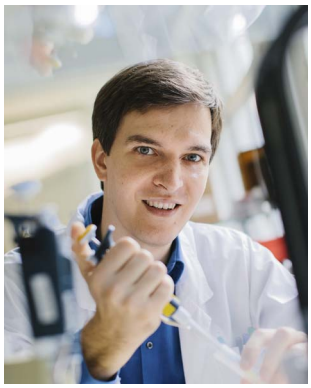

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1 Introduction

Bacillus subtilis is a Gram-positive bacterial species that belongs to the versatile *B. subtilis* group, along with *Bacillus pumilus*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens*.[1,2] It exhibits a remarkable genetic diversity which allows it to adapt to diverse ecological niches, ranging from deep-sea hydrothermal vents to the soil and the human gastrointestinal tract.[3,4] Its ability to form spores that are resilient to harsh conditions further contributes to the survival of *B. subtilis* in these challenging environments.[5] To gain a selective advantage in its particular habitat, *B. subtilis* also produces an extensive repertoire of bioactive metabolites, including polyketides (PKs), nonribosomal peptides (NRPs), ribosomally synthesized and post-translationally modified peptides (RiPPs) and terpenes with potent antimicrobial properties.[6,7] The species is also used as a biocontrol agent in agriculture for combatting plant pathogens and promoting plant growth.[6,8]

In industrial settings, *B. subtilis* has established itself as a reliable and versatile workhorse for the production of a wide array of compounds, ranging from enzymes to high-purity fine

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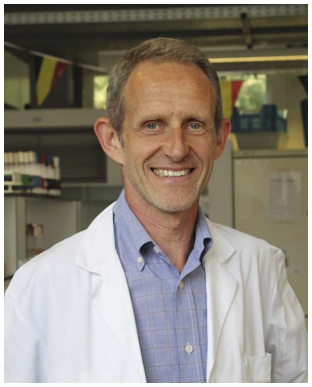
chemicals.[1,9,10] Its natural competence for DNA uptake,[11] high secretion capacity,[12] generally recognized as safe (GRAS) status,[13] lack of endotoxins,[14] and remarkable genetic diversity among closely related strains,[15] coupled with its well-described gene expression system[12] are all features that underpin its popularity.[16] In academia, *B. subtilis* continues to serve as a model organism for studying diverse physiological processes, such as protein secretion, cell motility and division, biofilm formation, minimal cell development, secondary metabolite biosynthesis, and molecular interactions with plants and fungi.[13,17]

Despite its promising biotechnological potential, the use of *B. subtilis* for heterologous secondary metabolite production lags behind other microbial hosts, such as *Escherichia coli*, *Streptomyces* spp. and *Saccharomyces cerevisiae*. However, over the past 15 years, significant advances have been made in the development of synthetic biology and genome engineering tools for *Bacillus* species.[11,14] With the growing availability of such tools, *Bacillus* is now gaining increasing interest as a heterologous host for natural product biosynthesis.[11] Heterologous expression is particularly valuable when tools for the genetic manipulation of the native host are limited or unknown, *e.g.*, for metagenome-derived gene clusters.[11] In the case of *B. subtilis*, successful heterologous production of NRPs,[18] PKs,[19] terpenoids,[14] and other small molecules and peptides[20,21] has been reported.

This review presents an overview of the natural products that have been successfully produced heterologously in *Bacillus* species, organized by natural product class. It also offers insights into the inherent advantages of *Bacillus* species as heterologous expression hosts and highlights diverse tools that can be employed for strain engineering and biosynthetic pathway manipulation. It explores potential biosynthetic gene clusters (BGCs) that may be suitable for expression in *Bacillus*, and provides recommendations for the most optimal (engineered) host strain for this purpose. With the ongoing advancement of molecular engineering tools, *B. subtilis* promises to emerge as a powerful player in the field of heterologous natural product biosynthesis.



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2 *Bacillus* as a host for heterologous expression of natural product BGCs

2.1 Favourable characteristics of *B. subtilis* for heterologous BGC expression

Various characteristics have been put forward to describe the ideal host strains for heterologous expression of natural product BGCs.[22–24] These include (1) easy cultivation and lab handling, (2) broad availability of gene editing tools and expression vectors, (3) thorough genetic characterization, (4) adaptable metabolism with adequate availability of (uncommon) precursors to support natural product biosynthesis, (5) clean genetic background, (6) promiscuous host transcriptional machinery, (7) high levels of self-resistance and efficient compound secretion systems, (8) ease of fermentation and upscaling, (9) Generally Recognized As Safe (GRAS) status and absence of endotoxins. In this section, we discuss these characteristics with respect to *B. subtilis* and other *Bacillus* spp., and compare them with other commonly used heterologous hosts, such as *Streptomyces* spp. and *E. coli*.

(1) Easy cultivation and efficient growth of the host strain are important factors for minimizing experimental workload and fermentation times. *B. subtilis* and *E. coli* both benefit from fast growth on standard nutrient agar, doubling in number every 20 to 30 minutes under ideal circumstances in the exponential phase. In contrast, *Streptomyces* strains take several days to form visible colonies. Handling Streptomycetes in the lab is also complicated due to their complex life cycle, which involves mycelial growth, hyphae formation, and sporulation.[25,26] For example, high-level expression of polyketide synthase (PKS) genes in *Streptomyces* typically occurs at the onset of the stationary phase of mycelial growth.[27] *B. subtilis* exhibits endospore formation, a well-studied trait regulated primarily by *spo0A* and triggered in response to starvation and other stresses.[28–30] These highly resistant spores facilitate survival in harsh conditions but may prevent cells from reaching high densities in bioreactors due to nutrient limitations in fed-batch

systems.[31] Several mutants have been constructed and tested to circumvent this challenge, as discussed in Section 4.1. The *Bacillus* life cycle also involves biofilm formation and swarming on surfaces. The former has been studied extensively in *Bacillus*[32] and a potential link between native secondary metabolism and biofilm differentiation has been proposed.[33] Intriguingly, heterologous production of the NRP iturin A in *B. subtilis* was found to be significantly enhanced during biofilm fermentation compared to planktonic cultures.[34] Notably, no biofilm-related problems have been reported during heterologous expression of BGCs in *Bacillus*. Swarming in *B. subtilis* has also been associated with the production of secondary metabolites, particularly biosurfactants like the cyclic lipopeptide surfactin.[35] However, swarming is primarily observed in undomesticated *Bacillus* strains due to frameshift mutations in the *sfp* and *swrA* genes in the majority of widely-used *B. subtilis* lab strains, *e.g. B. subtilis* 168.[36]

(2) A versatile set of genetic parts and gene editing tools is fundamental for the precise genomic integration and robust expression and engineering of natural product BGCs, which often span tens of kb in size and are frequently cryptic in nature. Like in *E. coli*, an elaborate molecular toolbox is available for *B. subtilis*, comprising standardized genetic elements,[37,38] a CRISPR-Cas system,[39] and markerless genome modification systems.[40–42] For a comprehensive overview of the *Bacillus* synthetic biology toolbox, the reader is referred to Section 4. The growing interest in *Streptomyces* as a host for natural product biosynthesis has recently fuelled the design and development of synthetic parts, chassis strains and genetic tools for this genus, bringing its toolbox closer to the level of more stablished model organisms. Recent examples include regulatory sequences for promoter engineering,[43] CRISPR-based tools for genome editing and silencing,[44,45] and specific vectors for targeted BGC capture, refactoring and expression.[46] Overall, *B. subtilis* offers a broader range of general engineering tools, while the tools available in Streptomycetes are more specifically designed for the expression of natural product BGCs. However, the cloning of BGCs and their introduction into the host remains a significant challenge. A particular advantage of *B. subtilis* over other hosts lies in its natural competence combined with its efficient homologous recombination machinery, allowing fast, straightforward and stable integration of large BGCs in the genome.[47] Contrary to *E. coli*, autonomous plasmids are unstable and prone to recombination in *B. subtilis*, especially when carrying very large inserts.[48–50] For a description of different cloning strategies for BGCs, the reader is referred to Section 4.3.

(3) To efficiently express natural product BGCs in a heterologous host, a thorough understanding of its metabolism and physiology is needed. Detailed analysis of the host's metabolic potential and genomic characteristics not only provides important information about precursor and cofactor availability, but also serves as a valuable resource for metabolic engineering, *e.g.* to limit the metabolic burden and enhance fermentation yields. The diversity and accessibility of genome modification tools in *B. subtilis* have substantially advanced its genetic characterization, making it one of the best-studied species to date. Nevertheless, despite decades of research, approximately one in four *B. subtilis* proteins remain uncharacterized.[51] The *MiniBacillus* project aims to reduce that number by creating a *B. subtilis* strain with a minimal genome. So far, approximately 42% of the genome has been successfully deleted as part of this initiative.[52,53] The absence of extracellular serine protease activity in this strain has enabled the successful production of subtilin, nisin and flavucin hybrids.[54] A comprehensive overview of *B. subtilis* genes, proteins and their interactions, expression networks, and other related information has been compiled in the *Subti*Wiki database.[17,55]

(4) Natural products are constructed from a diverse range of building blocks, which is reflected in their remarkable structural diversity and complexity. PKSs are known to use a variety of coenzyme A (CoA)-activated substrates, such as acetyl-, propionyl-, (methyl)malonyl-, and isovaleryl-CoA.[56] In *E. coli*, the metabolism controlling the fluxes of these substrates is under tight regulation, which can limit precursor supply during heterologous expression.[57] A variety of metabolic engineering strategies have been applied to increase cellular levels of acetyl- and malonyl-CoA in *E. coli*, including deletion of competing pathways and overexpression of the enzyme acetyl-CoA carboxylase, respectively.[58] The supply of methyl-malonyl-CoA, on the other hand, has been improved by expressing the *Streptomyces coelicolor* propionyl-CoA carboxylase and the *Propionibacteria shermanii* methylmalonyl-CoA mutase/epimerase pathways.[59] Nonribosomal peptide synthetases (NRPSs) are able to incorporate an even broader range of substrates, including (non-) proteinogenic amino acids, α-hydroxy acids and glycosylated and methylated residues. In *E. coli*, the number of native NRP pathways is limited, and they show only minimal diversity. Furthermore, heterologous NRP production in this species has been successful in only a limited number of cases.[60,61] Overall, the success rate for PK and NRP production in *E. coli* remains low, mainly because its metabolism is not well adapted for secondary metabolite production and because of problems with folding and stability of the large PKS and NRPS multienzyme complexes.[62–64] To enable post-translational activation of the acyl and peptidyl carrier proteins domains within PKSs and NRPSs, respectively, an engineered *E. coli* BAP-1 strain has been constructed, which expresses the *sfp* phosphopantetheinyl transferase gene from *B. subtilis*. Sfp is a phosphopantetheinyl transferase with an exceptionally broad substrate tolerance, capable of modifying ACP and PCP domains from almost any organism. Unlike *E. coli*, *B. subtilis* is a prolific producer of natural products, harbouring a great diversity of PK, NRP, and hybrid NRP-PK pathways.[65–68] While there is no doubt that *Streptomyces* strains contain the highest number of genomic BGCs on average, *Bacillus* species are not far behind. Nevertheless, genomic integration of additional precursor biosynthesis genes or feeding of uncommon amino acid building blocks has been proven necessary for the heterologous production of some NRPs.[18,69,70] For example, the supplementation of D-hydroxyisovalerate in the growth medium was shown to increase heterologous enniatin production,[69] while L-2,4-diaminobutyric acid feeding or heterologous expression of the ectoine biosynthetic gene *ectB* was required for heterologous polymyxin production.[18,70] Additionally, it is worth mentioning that several commonly used *B. subtilis* lab strains are

auxotrophic for specific amino acids. For example, *B. subtilis* 168 requires exogenously supplied tryptophan for growth.[71] Taken together, the metabolism of *B. subtilis* is well adapted for natural product biosynthesis, although feeding of specific building blocks is necessary in certain cases.

(5) While the native ability to produce many structurally diverse secondary metabolites signifies a well-adapted metabolism, it can also impede the production and detection of heterologous compounds. The metabolic burden associated with natural product biosynthesis is significant, and the presence of native compounds with similar biological activities can interfere with biological activity screenings. Heterologous hosts with 'clean' genetic backgrounds are therefore preferred. In Streptomycetes, such genome-reduced host strains, like *S. coelicolor* M1152 and M1154, have been successfully used for heterologous expression.[72–74] For *B. subtilis*, a markerless gene deletion system was used for genomic removal of all known antibiotic-producing BGCs, including those responsible for subtilosin, plipastatin, and bacilysin biosynthesis.[40] The resulting strain was evaluated for the production of surfactin. Although the growth rate was improved, overall surfactin biosynthesis was reduced compared to the control strain, and more research was deemed necessary to further optimize this strain.[75]

(6) Having promiscuous transcriptional machinery is beneficial for a heterologous host since it allows expression of diverse BGCs originating from a wide range of genetic backgrounds. The use of rare codons is one of the influencing factors in this context. *B. subtilis* is proposed to have a relatively low codon bias,[76,77] which in turn facilitates the expression of directly cloned BGCs. Additionally, *Bacillus* genomes have a low GC content. This makes cloning of BGCs faster and easier than in *Streptomyces* spp., whose genomes are very GC rich (>70% GC). This creates a specific niche for *B. subtilis* as a favourable host for the heterologous expression of low-GC BGCs.

(7) Efficient secretion of heterologously produced compounds and proteins allows for higher production titers and facilitates downstream purification processes. *B. subtilis* has a very efficient secretion system, comprising multiple pathways.[78,79] This feature has been exploited for industrial applications and is one of the reasons this species has become a popular workhouse for the industrial production of enzymes and chemicals. Prominent examples include α-amylases and riboflavin, produced through one of the most efficient fermentation processes in the world.[80,81] For heterologous production of secondary metabolites, which often have antimicrobial activity, rapid and efficient secretion is necessary to avoid potential toxicity to the host and to ensure self-resistance.

(8) Another quality of a good heterologous host is its compatibility with fermentation and upscaling processes. *B. subtilis*, as outlined above, has a long and successful track record of use in industrial settings. As a result, a wealth of experience has been gained in the upscaling of these processes.[12] Likewise, *E. coli* easily reaches high-cell density fermentations and the species has been widely used in industrial processes for the production of biofuels, amino acids, biopolymers, and many more.[82] While mycelial growth makes this process harder for Streptomycetes, effective industrial fermentation methods have also been established for this genus.[83]

(9) Lastly, host strains that do not produce endotoxins facilitate the use of fermentation in industrial production. *Bacillus* and *Streptomyces* both meet this criterion[84–86] in contrast to *E. coli*, whose lipopolysaccharides in the outer membrane need to be meticulously removed in a challenging and costly process.[87] Therefore, the RiPPs microcin J25 and Y were recombinantly produced in *Bacillus* instead of *E. coli*.[88] The absence of exotoxins and the non-pathogenic nature of *B. subtilis* has further led to the approval of the GRAS status for this species by the FDA.

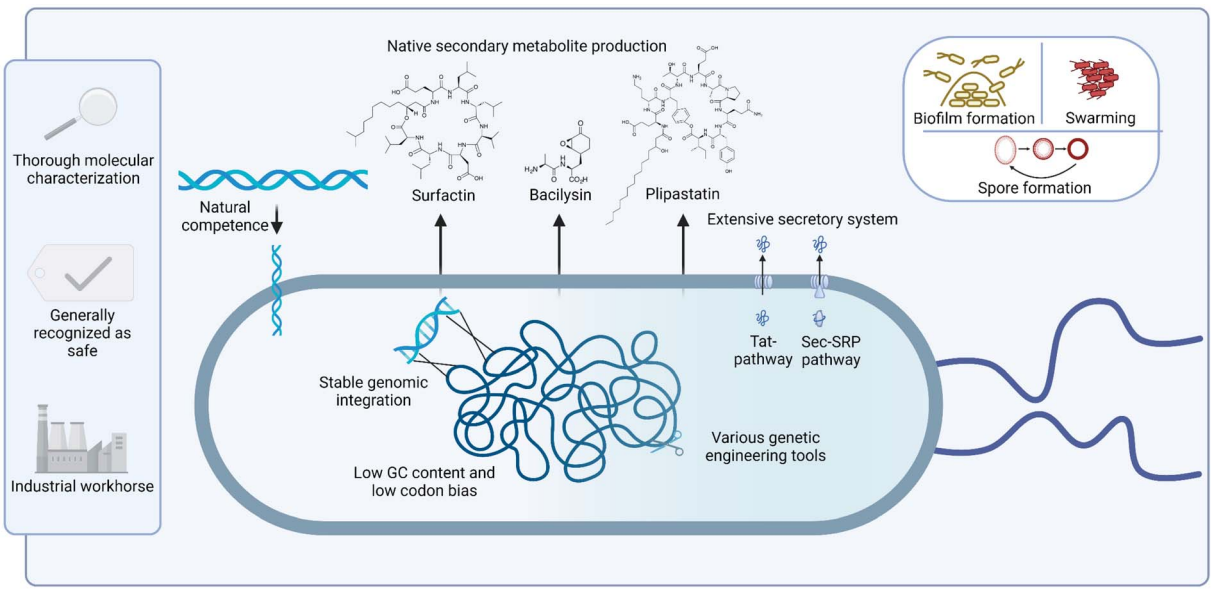


Fig. 1 Graphic representation of inherent advantages of using *B. subtilis* as heterologous production host for NPs. Figure created with BioRender, adapted from ref. 89–91.

In summary, *B. subtilis*, as non-pathogenic species, inherently possesses many traits that make it a favourable host for the heterologous expression of natural product BGCs (Fig. 1).[89–91] The combination of straightforward laboratory handling, decades of molecular characterization research and its natural competence and ability for natural product biosynthesis, suggest a promising future for this species as a host for heterologous BGC expression, a field that currently remains underexplored. The longstanding use of *Bacillus* species in industrial applications, along with the absence of exo- and endotoxins, and its extensive secretion system collectively highlight the potential in fermentation processes. Research gaps that remain to be studied include the design of more efficient expression systems for large BGCs, and the development of specific host strains with clean backgrounds for high yield secondary metabolite production.

2.2 BGC expression in other *Bacillus* species

Apart from *B. subtilis*, other *Bacillus* species have also been used for heterologous secondary metabolite production. A well-known example is *Bacillus velezensis* FZB42, formerly known as *Bacillus amyloliquefaciens* FZB42. This strain was originally isolated from the beet rhizosphere and its genome was first sequenced in 2007.[92] *B. velezensis* FZB42 is a model biocontrol agent and plant growth-promoting rhizobacterium. It shares many inherent characteristics with *B. subtilis*. Both are capable of sporulation and biofilm formation, have a natural competence for DNA uptake, possess efficient homologous recombination systems which facilitate genomic integration, and have received the GRAS status for specific industrial applications. The ongoing characterization of the strain has led to the creation of AmyloWiki, the *B. velezensis* counterpart of *Subti*Wiki, where information about gene functions, protein interactions and related details are collected.[93] Several genetic modification tools for this strain have been developed, including Cre-lox[94] and a CRISPR-Cas9 nickase.[100] However, they often suffer from low efficiencies. Different constitutive and inducible promoters have also been described,[96,97] but the development of other regulatory elements, such as ribosome binding sites (RBSs) and terminators, is lagging behind.[98]

Interest in *B. velezensis* as a host for heterologous production has mainly arisen from its extensive secondary metabolism, with over 10% of its genome dedicated to the synthesis of antimicrobial compounds.[99,100] Consequently, *B. velezensis* FZB42 has been used industrially as a biocontrol agent in different pesticides.[101] Successful examples of heterologous expression of BGCs in this strain remain scarce but have been reported in two studies. The first example is mersacidin, a lanthipeptide originally produced by a related *Bacillus* isolate. *B. velezensis* was chosen as a host because it already harboured a partial mersacidin (*mrs*) BGC, encoding an ATP binding cassette (ABC) transporter that provides self-resistance. The *mrs* BGC was completed *via* natural competence transformation and genomic integration of the missing genes, while the *mrsA* structural gene was expressed from an autonomously-replicating plasmid. Mersacidin production was confirmed through HPLC and MALDI-TOF analysis. Functional evaluation of heterologous mersacidin biosynthesis by monitoring the antibiotic activity of the *B. velezensis* host was more difficult due to the background production of other antimicrobial compounds.[102]

Another study has reported the heterologous expression of the BGC that directs the biosynthesis of the locillomycin lipopeptides.[99] This *loc* cluster originates from a closely related *B. velezensis* strain and was cloned in a fosmid vector. After exchanging the native promoter with an isopropyl β-D-1-thio-galactopyranoside (IPTG)-inducible P_{spac} promoter, the entire *loc* BGC was integrated in the genome of the host *via* homologous recombination, exploiting sequence homology in flanking genomic regions. RT-PCR analysis revealed that the expression pattern of the *loc* genes was comparable in both the wild-type strain and the heterologous host, with two-fold higher expression levels in the latter. Also here, biological activity testing of the locillomycin-producing host strain was hindered by native lipopeptide production. This indicates the importance of having host strains with a clean genetic background. Recently, a genome-reduced *B. amyloliquefaciens* strain that lacks several non-essential genes, including those involved in antibiotic production and prophage regions, was constructed.[103] This strain was shown to have a higher growth rate, reached higher biomass levels, and showed increased transformation efficiency. Surprisingly, however, no significant increase in surfactin production levels was observed. Nevertheless, this genome-reduced strain represents an interesting starting point for further engineering and optimization.

Bacillus licheniformis is another industrial workhorse that has been used for decades for the production of amylase[104] and the synthesis of bacitracin for veterinary applications.[105] The strain has similar characteristics as *B. subtilis*, but is particularly superior under anaerobic conditions. Several *B. licheniformis* strains have been isolated from oil reservoirs and have therefore adapted to oxygen-limiting conditions and higher temperatures.[106–109] An important factor behind this adaptation is the presence of an oxygen-independent ribonucleotide reductase, which is missing in *B. subtilis*.[110] This feature is especially interesting for the production of biosurfactants, like surfactin, fengycin, and lichenysin, which are natively produced by *Bacillus* species. Due to the need for oxygen supply in aerobic bioreactors, excessive foam formation occurs during surfactant production, a phenomenon that can be avoided under anaerobic conditions. An overview of the different tools and metabolic engineering strategies that have been applied to increase lichenysin surfactant titers in *B. licheniformis* was recently published.[111] Overall, heterologous BGC expression in this strain has garnered little interest up to now, even though it provides interesting opportunities for the production of oxidation-sensitive compounds.

3 Lessons from successful heterologous BGC expression in *Bacillus*

3.1 Nonribosomal peptides, polyketides and hybrids

NRPSs are large multimodular megaenzymes that operate in a stepwise assembly line fashion to construct structurally diverse peptide natural products. Each module consists of a set

of discrete catalytic domains responsible for the incorporation of one amino acid building block and a variable range of modifications to the growing peptide chain. Adenylation (A) domains select a specific amino acid building block, activate it *via* an adenylation reaction, and load it onto an adjacent thiolation (T) domain. T domains, also referred to as peptidyl carrier protein (PCP) domains, are post-translationally modified with a coenzyme A (CoA)-derived phosphopantetheinyl (Ppant) prosthetic group, which is required for covalent attachment of the aminoacyl and peptidyl intermediates. Following the loading reaction, condensation (C) domains couple the PCP-bound extender units to the growing peptide chain bound to the PCP domain of the preceding module by catalysing peptide bond formation. Throughout the assembly process, all building blocks and biosynthetic intermediates remain covalently bound to the PCP domains as thioesters. In most NRPSs, a terminal thioesterase (TE) domain catalyzes the release of the fully assembled peptide from the multienzyme complex *via* macrocyclization or hydrolysis. Additional domains, such as epimerization or methylation domains, and *trans*-acting tailoring enzymes can also be present, further increasing the structural diversity and complexity of NRPs.[112–114]

PKSs are another class of modular multienzyme complexes responsible for polyketide natural product biosynthesis. These biosynthetic assembly lines have evolved from fatty acid synthases and share functional and architectural similarities with NRPSs. In PKSs, acyltransferase (AT) domains specifically select small, CoA-activated acyl thioester starter and extender units, such as acetyl- and malonyl-CoA respectively, and load them onto the terminal thiol group of T domains, or acyl carrier protein (ACP) domains. Ketosynthase (KS) domains receive the growing polyketide chain from ACP domains in the upstream module and subsequently catalyse a decarboxylative Claisen condensation with the extender unit loaded by the AT domain. Additional auxiliary domains, such as ketoreductase, dehydratase, or enoyl reductase domains, can modify the α- and β-carbons in the ACP-bound intermediates, further increasing the structural diversity of polyketide natural products.[114,115] After the polyketide chain is fully assembled, the release of the product is frequently mediated by a TE domain which catalyses macrolactonization or hydrolysis. The resulting polyketide products can then undergo a range of post-assembly tailoring reactions, such as glycosylation, oxidation, hydroxylation, and halogenation, to attain their biological activity.

NRPSs, PKSs, and hybrid combinations of these biosynthetic assembly lines are designated as megasynth(et)ases. Common characteristics include the large size of these multifunctional enzyme complexes, the selection of specific building blocks, and the post-translational activation of the carrier protein (CP) domains with a Ppant prosthetic group in a reaction catalyzed by a 4′-phosphopantetheinyl transferase.[116] Successful heterologous expression of such BGCs in *B. subtilis* has been reported to varying extent for the different classes: expression of only two PKS BGCs has been described to date, in contrast to nearly a dozen PKS-NRPS hybrids and NRPSs (Table 1).

The polyketide erythromycin is a widely used broad-spectrum antibiotic, originally isolated from the actinomycete *Saccharopolyspora erythraea*.[117] 6-Deoxyerythronolide (6dEB) is the PK macrolide core of this compound, and the corresponding BGC has been heterologously expressed in *S. coelicolor*, *B. subtilis* and *E. coli*.[27,118,119] In *B. subtilis*, production levels only reached μg l^{-1} levels, while in the other hosts, much higher titers in the range of tens of mg l^{-1} were achieved.[19] Promising, however, is that production levels in the *Bacillus* fed-batch system remained high with stable cell densities, indicating that there is no lack of precursors in the cell and that proteins are expressed at constant levels. Furthermore, 6dEB was only detected in culture supernatants, and not in cell pellet extracts, which confirms that the compound is efficiently secreted out of the cell even in the absence of a dedicated transport gene in the BGC. Remarkably, 6dEB production in *B. subtilis* was detected only when each of the three core biosynthetic genes, ∼10 kb in size, were expressed individually from separate cassettes under the control of an acetoin-inducible promoter and with an optimized ribosome binding site. mRNA analysis suggested that the production issues were likely caused by mRNA instability due to secondary structures, rather than transcriptional challenges.

For the production of the NRP-PK hybrid amicoumacin, opposing results have been described. When the entire ∼47 kb amicoumacin BGC was expressed in *B. subtilis*, yields close to those of the native producer were achieved. In contrast, production levels in *E. coli* were 100-fold lower.[50] Furthermore, heterologous expression in *B. subtilis* under a strong, constitutive promoter of a PKS BGC from a *Blautia wexlerae* gut isolate resulted in the successful discovery of wexrubicin.[120] However, it should be noted that original producer and host strain were more closely related, with similar GC levels, in these last two cases. These examples indicate that heterologous PKS expression is feasible in *B. subtilis*, though it remains highly understudied.

Compared to PKS pathways, the successful heterologous expression of NRPS clusters in *B. subtilis* has been reported in multiple studies, spanning a diverse range of compounds, including bacitracin,[121] polymyxin A,[18] surfactin,[50] enniatin,[69] PZN12 pyrazonine,[122] plipastatin,[123] and bacillothiazols.[21] Additionally, *B. subtilis* has served as a host for the hybrid NRP-PK BGCs of iturin A[124] and bacillomycin D,[20] both BGCs composed of mostly NRP modules and only one PK module. The majority of these BGCs originate from species closely related to *B. subtilis*, and in these studies, the choice was made to clone the BGCs with their native promoters. Overall, the yields of the natural products in the *B. subtilis* host were comparable to those in the native producer or even exceeded them significantly. Edeine was one exception where, unexpectedly, no production could be detected in the host strain.[20] The authors suggested that the issue could lie with the supply of the uncommon building blocks spermidine and 1,3-diaminopropane, or with the potential inactivity of the native promoter from *Brevibacillus brevis* in the *B. subtilis* host strain. Generally, heterologous expression of closely related BGCs in *B. subtilis* has proven to be a successful strategy, giving rise to high production levels.

NRPS BGCs from taxonomically distant species have also been successfully expressed in *Bacillus*. Examples include the enniatin BGC from the fungus *Fusarium oxysporum*[69] and the

Table 1 Overview of natural products that have been heterologously produced in *Bacillus* species

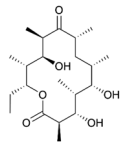
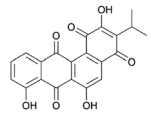
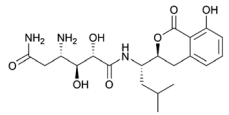
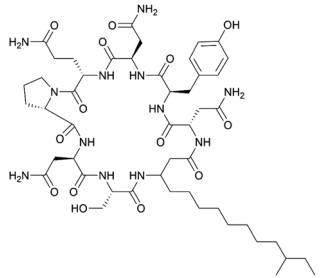
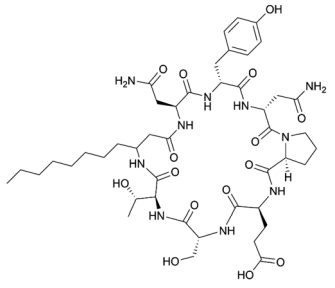
Compound	Class	Native producer	*Bacillus* host	BGC size	Yield	MiBIG	Ref.
6-Deoxyerythronolide B 	PKS	*Saccharopolyspora erythraea*	*B. subtilis* JK13 (168 derivative)	30 kb	2.6 ± 0.3 μg l^{-1}	BGC0000055	19
Wexrubicin 	PKS	*Blautia wexlerae* DSM 19850	*B. subtilis* 168-*sfp*	7.7 kb	0.06 mg l^{-1}		120
Amicoumacin 	NRPS/PKS	*Bacillus subtilis* 1779	*B. subtilis* JH642+*sfp*	47.4 kb	Comparable to native producer		50
Iturin A 	NRPS/PKS	*Bacillus subtilis* RB14	*B. subtilis* RM125 (168 derivative)	38 kb	51–64 μg ml^{-1}	BGC0001098	34 and 124
Bacillomycin D 	NRPS/PKS	*Bacillus velezensis* FZB42	*B. subtilis* 1A751	37.2 kb	Not reported	BGC0001090	20

Table 1 (*Contd.*)

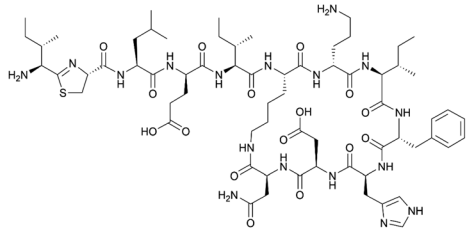
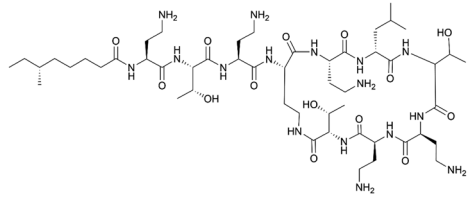
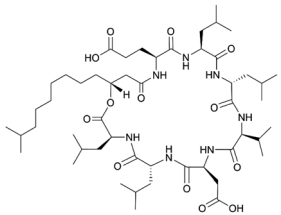
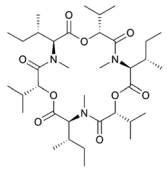
Compound	Class	Native producer	*Bacillus* host	BGC size	Yield	MiBIG	Ref.
Bacitracin 	NRPS	*Bacillus licheniformis* ATCC 10716	*B. subtilis* TS30 (ATCC 21332 derivative)	49 kb	Higher than native producer	BGC0000310	121
Two modules of surfactin BGC	NRPS	*Bacillus subtilis* ATCC 21332	*B. subtilis* KE30 (TS30 derivative)	6.2 kb	Not reported		64
Polymyxin A 	NRPS	*Paenibacillus polymyxa* E681	*B. subtilis* 168	40.6 kb	Not reported	BGC0000408	18, 70 and 128
Surfactin 	NRPS	*Bacillus subtilis* 1779	*B. subtilis* JH642	38 kb	Comparable to native producer		50
Enniatin 	NRPS	*Fusarium oxysporum*	*B. subtilis* 168	10 kb	1.1 mg l^{-1}	BGC0000342	69

Table 1 (*Contd.*)

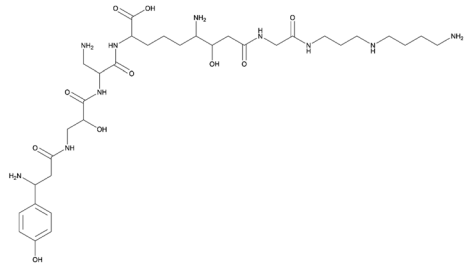
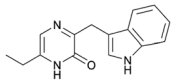
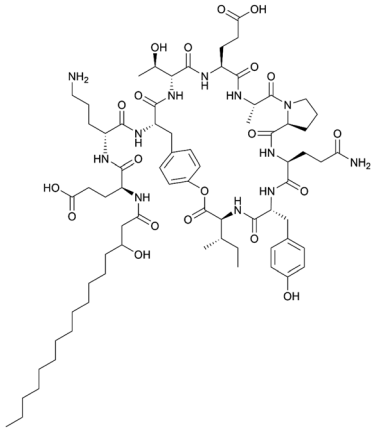
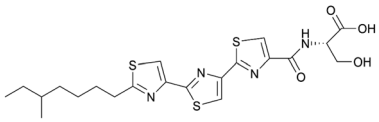
Compound	Class	Native producer	*Bacillus* host	BGC size	Yield	MiBIG	Ref.
Edeine 	NRPS	*Brevibacillus brevis* X23	*B. subtilis* 1A751	48.9 kb	Compound not detected		20
PZN12 	NRPS	*Blautia producta* ATCC 27340 and *Clostridium* sp. D5	*B. subtilis* 168	10 kb	Not reported		122
Plipastatin 	NRPS	*Bacillus amyloliquefaciens* HYM12	*B. subtilis* 1A751	38.4 kb	1182.5 ± 62.1 mg surfactin equivalents per l		123
Bacillothiazols 	NRPS	*Bacillus velezensis* FZB42	*B. subtilis* 1A751-*Sfp*	20 kb	Higher than native producer	BGC0002641	21

Table 1 (*Contd.*)

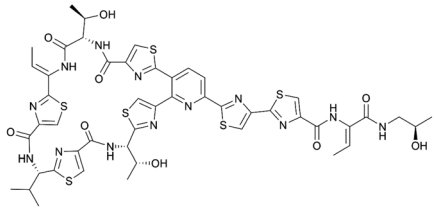
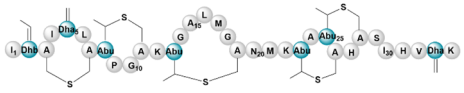
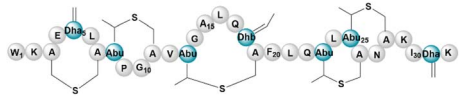
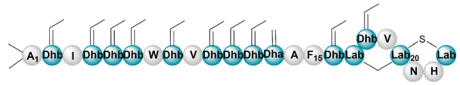
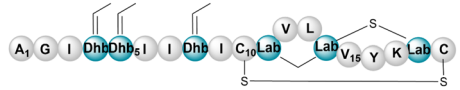
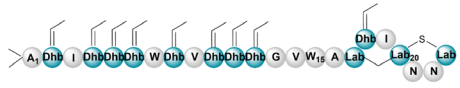
Compound	Class	Native producer	*Bacillus* host	BGC size	Yield	MiBIG	Ref.
Micrococcin P1 	RiPP (thiopeptide)	*Macrococcus caseolyticus* 115	*B. subtilis* BS 168R (168 derivative)	Not reported	Not reported		129
Nisin 	RiPP (lanthipeptide)	*Lactococcus lactis* ATCC 11454	*B. subtilis* 168 *ermΔsunA* (168 derivative)	16.5 kb	Only transcription	BGC0000535	130
	RiPP (lanthipeptide)	*Lactococcus lactis*	*B. subtilis* 168	Not reported	Only increase of resistance	BGC0000535	131
	RiPP (lanthipeptide)	*Lactococcus lactis* ATCC 11454	*B. subtilis* BRB779 (ATCC 6633 derivative)	Not reported	Not reported	BGC0000535	132
Subtilin 	RiPP (lanthipeptide)	*Bacillus subtilis* ATCC 6633	*B. subtilis* BR151 (168 derivative)	<45[a] kb	Not reported	BGC0000559	133
	RiPP (lanthipeptide)	*Bacillus subtilis* ATCC 6633	*B. subtilis* 168	12 kb	Comparable to native producer	BGC0000559	134
	RiPP (lanthipeptide)	*Bacillus subtilis* ATCC 6633	*B. subtilis* ATCC 6633, *B. subtilis* 168, *B. subtilis* WB800, *B. subtilis* 168 strain PG10	Not reported	Not reported	BGC0000559	54
Bacinapeptin A 	RiPP (lanthipeptide)	*Bacillus nakamurai* NRRL B-41092	*B. subtilis* 168	<15[a] kb	Not reported	BGC0002700	135
Paenithopeptin A 	RiPP (lanthipeptide)	*Paenibacillus thiaminolyticus* NRRL B-4156	*B. subtilis* 168	<15[a] kb	Not reported		135 and 136
Andalusicin A 	RiPP (lanthipeptide)	*Bacillus thuringiensis* sv. *andalousiensis* NRRL B23139	*B. subtilis* 168	Not reported	Not reported	BGC0002111	137
Subtilin-nisin hybrid	RiPP (lanthipeptide)	Not reported	*B. subtilis ATCC* 6633, *B. subtilis* 168, *B. subtilis* WB800, *B. subtilis* 168 strain PG10	Not reported	Not reported		54

Table 1 (*Contd.*)

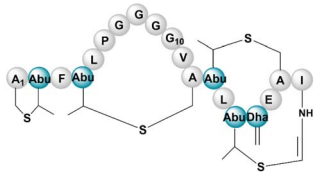
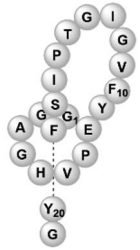
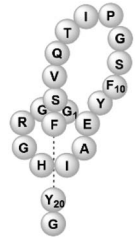
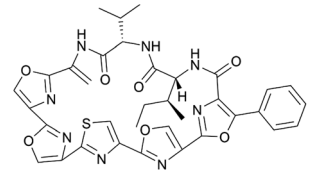
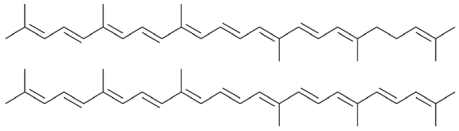
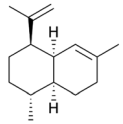
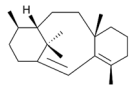
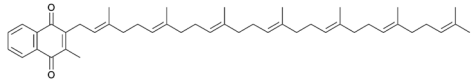
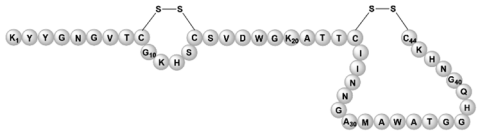
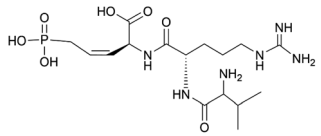
Compound	Class	Native producer	*Bacillus* host	BGC size	Yield	MiBIG	Ref.
Subtilin-flavucin hybrid	RiPP (lanthipeptide)	Not reported	*B. subtilis ATCC* 6633, *B. subtilis* 168, *B. subtilis* WB800, *B. subtilis* 168 strain PG10	Not reported	Not reported		54
Mersacidin 	RiPP (lanthipeptide)	*Bacillus* sp. HIL Y-8554728	*B. amyloliquefaciens* FZB42	12.3 kb	Not reported	BGC0000527	102
Microcin J25 	RiPP (lasso peptide)	Not reported	*B. subtilis* WB800N, *B. subtilis* 168	Not reported	5.968 mg l^{-1}		88
Microcin Y 	RiPP (lasso peptide)	Not reported	*B. subtilis* WB800N, *B. subtilis* 168	Not reported	3.303 mg l^{-1}		88
Mechercharmycin A 	RiPP (cyclic azol(in)e-containing peptide)	*Thermoactinomyces* sp. YM3-251	*B. subtilis* 168	Not reported	2–4 mg l^{-1}		138

Table 1 (*Contd.*)

Compound	Class	Native producer	*Bacillus* host	BGC size	Yield	MiBIG	Ref.
4,4′-Diaponeurosporene and 4,4′-diapolycopene 	Terpene (carotenoids)	*Staphylococcus aureus* NCTC 50581	*B. subtilis* JH642	Not reported	Higher than native producer		139
	Terpene (carotenoids)	*Staphylococcus aureus* NCTC 50581 and *Bacillus subtilis*	*B. subtilis* 168 *trp*C2	Not reported	10.65 mg g^{-1} dry cell weight		140
	Terpene (carotenoids)	*Staphylococcus aureus* NCTC 50581 and *Bacillus subtilis*	*B. subtilis* 168 *trp*C2	Not reported	21 mg g^{-1} dry cell weight		141
Isoprene 	Terpene (carotenoids)	*Bacillus subtilis* DSM 10	*B. subtilis* DSM 10 (ATCC 6051)	Not reported	Higher than native producer		142
Amorphadiene	Terpene (carotenoids)	*Bacillus subtilis* 1A1	*B. subtilis* 1A1	Not reported	20 mg l^{-1}		143
	Terpene (carotenoids)	Not reported	Not reported	Not reported	416 mg l^{-1}		14
	Terpene (carotenoids)	*Bacillus subtilis*	*B. subtilis* 168 *trp*C2	Not reported	116 mg l^{-1}		144
Taxadiene 	Terpene (carotenoids)	Taxadiene synthase from *Taxus baccata* and other genes from *Bacillus subtilis*	*B. subtilis* 168 *trp*C2	MEP pathway – 16.4 kb	17.8 mg l^{-1}		145
Menaquinone-7 (MK-7)	Terpene (carotenoids)	*Bacillus subtilis natto*	*B. subtilis* 168 *trp*C2	Not reported	69.5 mg l^{-1}		146
	Terpene (carotenoids)	Not reported	*B. subtilis* 168	Not reported	360 mg l^{-1}		147
Pediocin 	Bacteriocin	*Lactobacillus plantarum* Zhang-LL	*B. subtilis* WB800N, *B. subtilis* 168	Not reported	Not reported		148
Rhizocticin B 	Phosphonate oligopeptide antibiotics	*Bacillus subtilis* ATCC 6633	*B. subtilis* 168	13 kb	Not reported		149

[a] Exact size is not mentioned.

pyrazinone BGCs from *Blautia producta* and *Clostridium* gut species.[122] Although the production level of enniatin in *B. subtilis* was lower in comparison with that reported in the native producer,[125] it is remarkable that the expression of this native BGC was achieved in the heterologous host using an acetoin-inducible promoter. The pyrazinone clusters were codon-optimized and their expression was regulated by the hyper-P_{spac} promoter. Since the native producer strains for these BGCs were not available, these examples highlight the potential of synthetic biology in combination with heterologous expression of uncharacterized BGCs in *B. subtilis*, even from distantly related strains. However, it has to be noted that in the same study, five other uncharacterized BGCs were cloned analogously to the pyrazinone BGCs, but no heterologous products were detected. This reinforces the common notion that closely related heterologous hosts have a higher success rate.

3.2 Ribosomally synthesized and post-translationally modified peptides

RiPPs represent a group of post-translationally modified peptide natural products with highly diverse chemical structures and biological activities. RiPPs are first synthesized by ribosomes as inactive, linear precursor peptides consisting of a leader and a core region. RiPP biosynthesis involves recognition of the leader peptide by enzymes that post-translationally modify the core peptide. In the final step, the precursor peptide is typically processed by a protease that cleaves off the leader peptide. The modified and bioactive core peptide is then exported out of the cell.[126,127]

Although *E. coli* and *Streptomyces* spp. are currently the most commonly used heterologous expression hosts for RiPP BGCs,[126] *Bacillus* species have been put forward as a promising alternative for various reasons. First, a wide range of native RiPP BGCs have been discovered in the genome of *Bacillus* spp., indicating that this genus is well equipped to produce this class of compounds.[150,151] This proficiency has been demonstrated by the successful recombinant production of micrococcin P1, a thiopeptide produced by *Macrococcus caseolyticus* 115. $tRNA^{Glu}$ played a pivotal role as a glutamate donor during the biosynthesis of this RiPP. Since the sequence of $tRNA^{Glu}$ from *B. subtilis* BS 168R differed by only a single base pair from that of the original host, this *B. subtilis* strain was considered to be the ideal heterologous host for the purpose of discovering essential biosynthetic genes and studying promoter activities within the micrococcin P1 BGC.[129] Secondly, *Bacillus* has been used for the heterologous production of RiPPs to study their biosynthetic pathway or examine the effect of regulators on pathway expression when genetic modification in the original, closely-related host was difficult.[102,129,134]

The majority of RiPP BGCs are introduced into the genome of a *Bacillus* host through homologous recombination, although some are expressed in *trans* from a plasmid. One method that has been used to genomically integrate RiPP BGCs involves fragmenting the genomic DNA and adding homologous arms to incorporate the genomic region of interest into the genome of the heterologous host. Examples that have used this technique include the heterologous production of the broad-spectrum lanthipeptides subtilin[133] and nisin[130] in *B. subtilis* BR151 and *B. subtilis* 168, respectively. However, this method has yielded mixed results. In the case of subtilin, recombinant production was successful,[133] while for nisin, BGC expression was verified by RT-PCR, but the active RiPP could not be detected.[130] A possible explanation for this discrepancy is the absence of a nisin resistance mechanism in the host. In one study, this problem was solved by engineering *B. subtilis* 168 to increase its resistance in two ways. First, self-resistance genes found in the nisin BGC were integrated into the genome of *B. subtilis* 168 under the control of a synthetic promoter. Secondly, genes associated with nisin resistance were identified *via* transcriptomics and proteomics analysis of nisin-treated cells. The genes upregulated in this experiment were overexpressed in *B. subtilis* 168 to enhance its nisin resistance. Most of these genes were related to a general cell surface or membrane stress response rather than being specific to nisin. However, despite these efforts, the resistance of this engineered *Bacillus* host was not sufficient to allow commercial production of nisin.[131] In another study, switching to a heterologous host that naturally possessed resistance to nisin enabled heterologous production.[132] The same technique was also applied for fruitful recombinant production of the lanthipeptide mersacidin, which is naturally synthesized by *B. velezensis*.[102]

In addition to increasing host resistance, engineering the regulatory mechanisms that control the expression of RiPP BGCs has had a positive impact on yield. For example, production of the lanthipeptide subtilin is repressed during the exponential growth phase due to the presence of the transition state regulator AbrB. Deletion of the gene encoding this regulator led to a remarkable increase in recombinant subtilin production. Moreover, altered medium composition further increased the production levels to yields comparable to those of the wild type.[134]

The heterologous expression of RiPP BGCs in *Bacillus* has also led to the discovery of novel class III lanthipeptides from *Firmicutes*.[135–137] Most of these lanthipeptide BGCs lack a protease gene. Through a combination of correlation network and co-expression analysis, possible proteases for these lanthipeptides were predicted. As a proof of concept, two previously unreported class III lanthipeptide BGCs were heterologously expressed in *Bacillus*, together with their predicted protease genes. This approach resulted in the discovery of bacinapeptins A and B, and paenithopeptins A-E[136]. Additionally, heterologous expression in *Bacillus* revealed an uncommon N-terminal dimethylation in a subset of class III lanthipeptides.[136,137] This modification was linked to a methyltransferase that utilizes *S*-adenosyl methionine as a donor. This dimethylation, found in andalusicin[137] and variants of paenithopeptin,[136] was found to increase their antibacterial activity[136,137] and is dependent on specific amino acids present at the first two positions of the core peptide.[136]

RiPPs show a remarkable biosynthetic malleability thanks to the tolerance of many RiPP modifying enzymes to amino acid changes in the core peptide sequence. The organization of precursor peptides in two distinctive parts (leader and core

peptide) greatly facilitates this promiscuity as it allows leader peptide-dependent tailoring enzymes to remain substrate-specific while processing a wide variety of core sequences. Generating RiPP analogues by implementing these principles has led to the discovery of RiPPs with completely new characteristics and functions. For an overview of the engineering potential of RiPPs, the reader is referred to interesting reviews by Hudson and Mitchell (2018) and Montalbán-López *et al.* (2021).[152,153] Various studies have taken advantage of this relaxed substrate specificity during heterologous expression in *Bacillus*. In a first study, nisin was recombinantly produced using the subtilin modification machinery present in the host strain. Therefore, the *nisA* gene encoding the nisin precursor peptide was introduced into the *Bacillus* genome containing the subtilin modification machinery. To ensure leader peptide recognition by this machinery, a part of the nisin leader peptide was replaced with that of subtilin, encoded by the *spaS* gene. However, most of the original nisin leader peptide needed to be retained to ensure efficient nisin production.[132] In a second study, this principle was further exploited to develop a robust platform for the production of various unnatural lanthipeptide analogues. The subtilin modification and transport machinery, encoded by *spaBTC*, were combined with hybrid peptides composed of the leader peptide of subtilin, encoded by *spaS*, and the core peptide of either subtilin, nisin, or flavucin, encoded by *spaS*, *nisA* and *flaA*, respectively. Additionally, the subtilin leader peptide cleavage site was replaced with that of nisin to prevent *in vivo* leader peptide removal and resulting toxicity of the active RiPP. This approach resulted in a higher yield of inactive lanthipeptides, which were subsequently subjected to *in vitro* leader peptide removal for activation.[54]

Bacillus has also served as a heterologous host for RiPP BGCs from taxonomically unrelated species. Examples include the lasso peptides microcin J25 and microcin Y originating from Enterobacteriaceae. Together, they exhibit antibiotic activity against food-born *Salmonella*. To optimize their production in *Bacillus*, the difference between expression from a plasmid and the integration of one or more copies of the BGC in the genome was evaluated. In the end, production of both microcins was achieved with comparable yields to *E. coli*.[88] Another example involves mechercharmycin A, a recently discovered polyazole cyclopeptide naturally produced by a *Thermoactinomyces* sp. This azol(in)e-containing RiPP exhibits strong cytotoxicity against various cancer cell lines. To discover more about the biosynthetic pathway of this RiPP, its BGC was integrated into the *Bacillus* genome under the control of a strong constitutive promoter, while an extra copy of the mechercharmycin A precursor peptide gene was expressed from a plasmid. A yield of 2–4 mg l^{-1} was achieved, surpassing the yield of 0.035 mg l^{-1} obtained from the native producer.[138]

In summary, heterologous production in *Bacillus* has been established for certain classes of RiPPs, including thiopeptides, lanthipeptides, lasso peptides, and azol(in)e-containing RiPPs. However, it remains largely unexplored territory for the majority of RiPP families. Successful recombinant production has been achieved for compounds originating from closely related species, as well as from some more distantly related ones. Key factors that need to be taken into account in this process are immunity of the host strain to the RiPP being produced and the regulatory mechanisms governing RiPP BGC expression.

3.3 Terpenoids

Bacillus has proven particularly successful as a heterologous host for terpene and terpenoid biosynthesis, owing to its inherent ability to produce isoprene in high concentrations *via* the methylerythritol phosphate (MEP) pathway. This pathway leads to the production of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which serve as the precursors for most terpenoids. By combining this inherent characteristic with the introduction of relevant terpene synthases and metabolic engineering optimizations, *B. subtilis* has been engineered to produce a variety of terpenoids, including carotenoids, amorphadiene, taxadiene, and menaquinone-7.[144] It is worth noting that while *Bacillus* spp. natively possess the MEP pathway, other Gram-positive cocci and *Lactobacillus* spp. exclusively rely on the mevalonate (MVA) pathway, while *Listeria* spp. and a subset of Actinobacteria, such as *Streptomyces*, utilize both the MVA and MEP pathway.[14,154–160]

As *B. subtilis* is recognized for its ability to produce significant quantities of isoprene, it has long been considered a promising microbial platform for terpenoid production. However, the progress in developing *B. subtilis* as a robust terpenoid cell factory has lagged behind that of *E. coli* and *S. cerevisiae*, primarily due to the slower development of molecular tools tailored specifically for *B. subtilis*. However, recent studies focusing on *B. subtilis* have yielded promising results, underscoring its potential as an efficient terpenoid production platform (Table 1).[14]

Carotenoids are widely used in the food, pharmaceutical, and health protection industries and researchers have turned to microorganisms for their production. Early metabolic engineering efforts in *B. subtilis* involved the introduction of two genes from *Staphylococcus aureus* (*crtM* and *crtN*) to produce C30 carotenoids, enhancing the organism's resistance to oxidative stress.[139] The enzyme CrtM catalyzes the transformation of farnesyl pyrophosphate (FPP) into dehydrosqualene. Subsequently, CrtN, acting as a dehydrosqualene desaturase, facilitates the conversion of dehydrosqualene into a vibrant yellow C30 carotenoid, known as 4,4′-diaponeurosporene.[139] Later engineering strategies focused on increasing isoprene production and overexpressing the 1-deoxy-D-xylulose-5-phosphate synthase (*dxs*) gene, which is responsible for condensing pyruvate and glyceraldehyde-3-phosphate (G3P) to form 1-deoxy-D-xylulose-5-phosphate (DXP). DXP is subsequently processed into IPP and its isomer DMAPP.[142] Further improvements were achieved by overexpressing multiple MEP pathway genes, resulting in high levels of C30 carotenoid production in *B. subtilis*.[140] The upregulation of the whole MEP pathway further increased carotenoid production by up to around 20 mg g^{-1} dry cell weight.[141]

The crucial precursor for the highly effective antimalarial drug artemisinin is amorphadiene. Currently, microbes are responsible for producing this precursor, which is then converted into artemisinin through chemical methods. Researchers

have constructed the amorphadiene biosynthetic pathway in *B. subtilis* by co-expressing the amorphadiene synthase gene (*ads*) with *dxs* and *idi*, two genes in the DXP pathway.[143] ADS is required to convert FPP into amorphadiene, while Idi is essential in balancing the high flux of IPP generated by the MVA pathway.[161] Increasing ADS translation and the expression of active terpene synthases can significantly improve production.[143] *B. subtilis* has shown promising results in producing the artemisinin precursor (416 mg l^{-1}) through growth medium optimization and additional expression of the FPP synthase gene *ispA* and the entire MEP pathway.[14] In a parallel study, a CRISPR-Cas9 editing system has been used to engineer three additional modules for improving amorphadiene production. This involved enhancing the MEP pathway, mutagenizing the terpene synthase module for overexpression, attenuating the branch pathway module and regulating the TCA cycle metabolism through the use of various weak and strong promoters. These modifications increased extracellular amorphadiene production from 81 to 116 mg l^{-1}.[144]

Taxadiene is the crucial precursor for paclitaxel, a well-known anticancer drug marketed under the brand name Taxol. In *B. subtilis*, functional production of taxadiene was achieved by overexpressing the complete MEP pathway, along with the introduction of the taxadiene synthase (Txs) enzyme. Co-expression of the geranylgeranyl pyrophosphate synthase gene *crtE* from *Pantoea ananatis* led to 17.8 mg l^{-1} of taxadiene in *B. subtilis*, surpassing the yield achieved in yeast.[145] Fine-tuning the expression of the MEP pathway genes in *E. coli* resulted in even higher yields. This success has inspired further improvements in *B. subtilis* taxadiene production through promoter and RBS optimization.[14,162]

MK-7, a major vitamin K2 compound recognized for promoting bone growth and cardiovascular health, has traditionally been produced through the fermentation of *B. subtilis natto* strains.[163] However, researchers have engineered *B. subtilis* 168 for significantly increased MK-7 production by overexpressing endogenous modular pathways (MK-7, shikimate, MEP, and glycerol metabolism). A yield of 12.0 mg l^{-1} of MK-7 was achieved through the overexpression of the 1,4-dihydroxy-2-naphthoyl-CoA hydrolase gene (*menA*) under the control of the P_{laps} promoter, along with four MEP pathway genes. Production levels were further increased by enhancing glycerol metabolism and reducing intermediate metabolite consumption.[14,146] The integration sites for overexpression of the MEP pathway genes were also found to affect MK-7 production.[146] Finally, dynamic regulation of pathway expression through quorum sensing led to a remarkable increase in production, reaching 360 mg l^{-1} in *B. subtilis*, the highest reported yield.[147]

While substantial progress has been made toward optimizing terpenoid production in *B. subtilis*, there is still ample room for further improvement. First of all, there is an opportunity to harness the inherent assets that *B. subtilis* possesses, such as cytochrome P450s and glycosyltransferases, to further diversify the range of terpenoids produced.[14] Secondly, current engineering efforts primarily focus on overexpressing genetic components of the pathway due to limited information on the steady-state kinetic parameters of the endogenous enzymes. While this approach is common in metabolic engineering, it can impose a burden on the cells and incur energy costs.[164] Protein engineering techniques, such as directed evolution, can enhance enzyme catalytic activity and reduce negative feedback inhibition.[161] Promiscuity in terpene synthases can also be minimized to streamline precursor utilization and reduce metabolic burden. In addition, protein fusions and synthetic protein scaffolds can be employed to improve enzyme expression, solubility, stability, and localization to substrates or cofactors.[165–168] Therefore, a holistic approach involving multilevel engineering, including gene expression manipulation, protein engineering, and comprehensive omics data analysis, is necessary. Collectively, these strategies will be essential for developing optimal *B. subtilis* strains for terpenoid production.[14,169]

3.4 Other bioactive natural products

Most heterologous expression efforts have focused on well-known classes of natural products such as RiPPs, NRPs, PKs, and terpenoids. However, *B. subtilis* has also proven to be a suitable platform for the production of less-known natural product classes. For example, pediocin, a group IIα bacteriocin with antimicrobial activity against *Listeria* spp., is naturally produced by *Pediococcus acidilactici* and *Lactobacillus plantarum*.[170,171] Heterologous production of this bacteriocin has been achieved in various hosts, such as *E. coli*,[172,173] *Lactobacillus lactis*,[174,175] and *Corynebacterium glutamicum*.[176] A study by Wang *et al.* investigated if the pediocin peptide (PapA) could be heterologously produced in *B. subtilis* WB800N and secreted without the need for accessory protein PedC/PapC and ABC transporter PedD/PapD, as previous research had shown that these proteins impose a metabolic burden to the host[148,176] (Table 1). The study used a shuttle vector to express both the original and a codon-optimized version of the *papA* gene as a fusion protein with an α-amylase signal peptide for secretion.[177–179] The resulting supernatants, obtained after induction of expression, both displayed anti-*Listeria* activity, confirming successful secretion and bioactivity of the fused pediocin peptides. This finding was significant as it represented the first time actively secreted pediocin was obtained without the presence of the cognate secretion machinery. Notably, these results differed from previous findings in *E. coli*., where the fused protein did not exhibit any antibacterial activity.[172,173,180] Surprisingly, the inhibitory activity of the non-codon-optimized fused peptide was stronger than that of codon-optimized one. The authors speculated that the difference in codon usage did not significantly affect expression levels in *B. subtilis*, at least for the *papA* sequence. They acknowledged the need for future research to explore the influence of the His-tag on the anti-*Listeria* activity and to optimize the induction conditions for pure PapA/pediocin. The pediocin produced in *B. subtilis* showed similar thermotolerant properties as reported in previous studies.[148,181,182]

Rhizocticins are antifungal phosphonate oligopeptide antibiotics produced by *B. subtilis* ATCC 6633 (Table 1).[149,183] These antibiotics consist of di- and tripeptides with the unique non-proteinogenic amino acid (*Z*)-L-2-amino-5-phosphono-3-

pentenoic acid (APPA) at their C-terminus. Rhizocticins enter fungal cells through an oligopeptide transport system. Within the cell, they are cleaved by fungal peptidases to release APPA, which inhibits threonine synthase, leading to inhibition of protein synthesis and growth.[184,185] Similarly, plumbemycins, tripeptide antibiotics with a C-terminal APPA residue from *Streptomyces plumbeus*, also use an oligopeptide transport system to enter bacterial cells and inhibit cell growth by releasing APPA. The selectivity of these antibiotics is determined by the recognition of the specific amino acids attached to APPA by the transport system and peptidases. Since threonine synthase is not present in mammals, the cytotoxicity of these antibiotics is limited.[149,186–188] While the rhizocticins where discovered in 1949,[183] the rhizocticin biosynthetic cluster was only identified in the genome of *B. subtilis* ATCC 6633 in 2010 *via* genome mining. The *rhi* cluster was heterologously expressed in *B. subtilis* 168 and experimentally shown to produce rhizocticin B using a combination of phosphorus-31 (^{31}P) NMR spectroscopy and LC-MS.[149] To date, only a few phosphonate biosynthetic pathways have been studied in detail.[149] Elucidating and understanding the biosynthetic pathways of phosphonates can offer valuable insights into unique enzymatic transformations and novel biochemistry.

These examples demonstrate that *B. subtilis* can also be used to produce less well-known classes of natural products. However, the suitability of *B. subtilis* as an expression host for such metabolites needs to be assessed on a case-by-case basis.

4 *B. subtilis* toolbox for strain and biosynthetic pathway engineering

4.1 Host strain engineering

4.1.1 Genome modification and metabolic engineering. Different strategies have been implemented to improve the host characteristics of *B. subtilis*, facilitate its lab handling, and increase the production levels for heterologous compounds. In this section, we will discuss genomic modifications to various regulatory genes and pathways (Table 2), as well as metabolic engineering approaches aimed at achieving these objectives.

4.1.1.1 DNA uptake. Due to its natural competence, transformation of *B. subtilis* requires relatively little effort. However, the presence of the intrinsic *BsuM* restriction–modification (R–M) system in the widely used lab strain *B. subtilis* 168 causes plasmid instability and reduced transformation efficiencies for foreign DNA and large sequences.[189] The process of cloning and heterologously expressing BGCs, which commonly faces these challenges, therefore remains a challenging task. Encouragingly, deletion of the *ydiO-ydi*P and *ydiR-ydiS-ydjA* operons, thereby disrupting the R–M system, has been reported to increase transformation efficiencies for 30–70 kb fragments by at least 100-fold.[18] A complementary strategy involves engineering the *comSK* system.[190] Overexpressing these central competence regulator genes under the control of the mannitol-inducible P_{mltA} promoter has been shown to increase transformation efficiencies of plasmid DNA by 6.7 fold, resulting in a so-called super-competent *B. subtilis* 168 derivative.[191] Another strain, *B. subtilis* JK3, in which *comS* was placed under the control of an IPTG-inducible P_{spac} promoter in the *sacA* locus, was used for heterologous enniatin production, but no quantification of competence was described.[69] Both of these competence-enhancing approaches seem valid to use in *B. subtilis* host strains to facilitate cloning.

4.1.1.2 Sporulation. To improve lab handling, boost production levels and increase cell densities in bioreactors, efforts have been made to disable the sporulation pathway. However, the outcomes have been mixed. Knocking out the *spo0A* gene, which encodes a general regulator for initiating the sporulation process,[28] resulted in reduced heterologous production of subtilin[134] and even complete abolition of heterologous polymyxin production.[70] Modification of the signal-transducing protease gene *spoIIGA*, on the other hand, increased levels of heterologous 6dEB production,[19] but unexpectedly led to significantly reduced enniatin titers.[69] Notably, significantly reduced cell density levels were reported in both publications, while the *spoIIGA* deletion in the undomesticated *B. subtilis* ATCC 6051 strain resulted in increased cell densities.[192] In a recent study, the effect of single sporulation gene deletions on enzyme and secondary metabolite production levels in *B. subtilis* was investigated.[193] It was found that single deletions of either *spo0A*, *spoIIIE*, or *spoIVB* were sufficient to abolish sporulation, but each had different effects on physiology and production. Specifically, native surfactin production was abolished in the *spo0A* mutant but increased in the other two mutants. Since the *spoIIIE* deletion compromised cell integrity, the *spoIVB* mutant was identified as the best secondary metabolite producer. The positive effect was suggested to be linked to metabolism, with increased synthesis of branched-chain fatty acids, glutamate, and aspartate, even though valine and leucine levels were decreased. Furthermore, *spo0A* deficient strains show greatly reduced competence levels.[194] Partial reversal of this effect can be achieved through the modification of AbrB, a small regulatory protein negatively regulated by Spo0A.[201] AbrB has been shown to downregulate or abolish the expression of heterologous BGCs,[134] even by directly binding upstream of the heterologous polymyxin BGC, native to *Paenibacillus* spp.[70] Both studies suggest, through the analysis of double mutants, that modifying *spo0A* leads to derepression of *abrB*, which, in turn, results in reduced production levels. The combination of a *spo0A* nonsense mutation and a stop codon mutation in *abrB*, leading to an 11 bp extension of the open reading frame, is naturally present in *B. subtilis* 3NA.[202] The effect of these mutations on surfactin production was recently studied in a *B. subtilis* 168-derived strain. The findings indicated that *spo0A* mutants can reach higher cell densities with low surfactin production, while *abrB*-elongated mutants attained lower cell densities but high surfactin production. The combination of both modifications had overall beneficial effects.[194]

4.1.1.3 Biofilm formation. Biofilm formation is another trait of *Bacillus* that can complicate lab handling and requires significant metabolic capacity. The deletion of the *epsA-O* and *tasA-sipW-yqxM* operons, responsible for biofilm formation,[32] leads to increased levels of native surfactin production in *B.*

Table 2 Overview of genes that have been modified during host strain engineering of *B. subtilis*

Pathways/gene functions	Genes	Effect on NP production	Other effects	Ref.
Natural competence	BsuM restriction/ modification system	—	KO increases transformation efficiency	18
	comSK	—	Induced expression increases transformation efficiency	69 and 191
Sporulation	*spo0A*	KO decreases heterologous subtilin production, and abolishes heterologous polymyxin production and native surfactin production	KO abolishes sporulation and reduces competence levels	70, 134, 193 and 194
	spoIIGA	KO decreases heterologous enniatin production and increases heterologous 6-deoxyerythronolide B production	KO abolishes sporulation and affects cell density	19, 69 and 192
	spoIIIE, *spoIVB*	KO increases native surfactin production	KO abolishes sporulation and cells lack cell integrity	193
General regulator	*abrB*	KO increases heterologous polymyxin and subtilin production. *abrB* elongation increases native surfactin production	*abrB* mutants reach lower cell densities	70, 134 and 194
	degQ	DegQ is essential for native plipastatin production. Functional DegQ increases heterologous iturin, heterologous plipastatin and native fengycin production, and decreases native surfactin production	KO or downregulation of *degQ* reduces protease activity	123, 124, 195 and 196
Biofilm	*epsA-O tasA-sipW-yqxM*	KO increases native surfactin and fengycin production	KO decreases biofilm formation	197 and 198
Autolysis	*lytC*	KO increases heterologous enniatin and 6dEB production	KO reduces autolysis and increases cell densities	19, 69 and 192
Protease activity	*nprE*, *aprA*	Successful heterologous production of bacillomycin D, plipastatin and bacillothiazols in KO strain	—	20, 21, 123 and 199
	nprE, *aprE*, *epr*, *bpr*, *mpr*, *nprB*, *vpr*, *wprA*	Successful heterologous production of microcins in KO strain	—	88 and 200
PPTase	*sfp*	Sfp is essential for the production of native lipopeptides. It is compatible with a wide range of heterologous NRPSs and PKSs	—	18–20 and 50

subtilis by allowing the reallocation of energy and substrates.[197] For native fengycin titers, only the *tasA* deletion has been shown to improve production, while the other mutants did not show significant differences.[198]

4.1.1.4 Autolysis. Additional yield improvements for secondary metabolites have been achieved by deleting *lytC*, which encodes an autolysin responsible for flagellar function. Inactivation of *lytC* increased heterologous enniatin and 6dEB production.[19,69] Furthermore, this modification is associated with reduced autolysis and the ability to reach higher cell densities compared to the wildtype strain.[192]

4.1.1.5 Growth phase regulation and proteolysis. The DegS–DegU two-component system in *B. subtilis* is involved in regulating the transition to the stationary growth phase, affecting various processes, including competence initiation, motility, and poly-γ-glutamate synthesis.[203] DegQ promotes the activation of this system *via* phosphorylation reactions, and this regulatory protein is therefore essential for plipastatin production in *B. subtilis* 168.[204,205] Functional expression of DegQ also led to increased production levels in a *B. subtilis* 3NA derivative, but interestingly, promoter exchange in the plipastatin BGC to the strong constitutive P_{veg} promoter abolished this increase.[195]

The native *degQ* gene in the *B. subtilis* 168 lab strain has a one base pair substitution in the promoter region, causing greatly reduced expression of the gene.[196,206] The impact of *degQ* on secondary metabolite production appears to be variable. Heterologous production of the NRP iturin increased to half of the wildtype producer levels upon $degQ_{YB8}$ expression[124] and *degQ* expression was also successfully used for heterologous plipastatin production.[123] However, for the production of native lipopeptides, detrimental effects were observed for surfactin levels, while fengycin production was high.[196] Additionally, the direct effect of DegQ on the production of extracellular proteases was analyzed. This is especially important for RiPP-like natural products to avoid degradation. No effect on NRP-derived lipopeptides was detected, but *degQ* downregulation or deletion significantly reduced protease activity.[196] An alternative and more straightforward approach to avoid proteolysis is the use of protease-deficient heterologous hosts. A well-known example is the two-protease deficient *B. subtilis* 1A751 strain,[199] which has been used for heterologous expression of several different classes of BGCs.[20,21,123] Another option is the WB800 strain, in which eight proteases have been disrupted[200] and successful heterologous microcin production has been achieved.[88]

4.1.1.6 PKS and NRPS biosynthesis. Phosphopantetheinyl transferases are essential for the posttranslational modification and functionality of PKSs, NRPSs, and fatty acid synthases by converting the inactive *apo*-enzymes to their active *holo* forms. In *B. subtilis*, the native *sfp* gene encodes such an enzyme, which has served as a model for the entire enzyme class due to its widespread use and remarkable substrate promiscuity. Sfp is essential for the production of the native lipopeptides surfactin and plipastatin in *B. subtilis*.[204,207] However, the most widely used lab strains, like *B. subtilis* 168, contain a frameshift mutation in the *sfp* coding sequence, resulting in premature translation termination.[20] To overcome this problem, functional *sfp* genes from other *Bacillus* species have been integrated into the genome,[18,50] or the frameshift mutation in the native gene has been reversed.[19,20] Sfp is able to interact productively with an exceptionally broad range of NRPSs and PKSs from both Gram-positive and -negative bacteria, making it a crucial component of a heterologous expression platform.

In order to increase the production levels of secondary metabolites, metabolic engineering is a valid strategy. Even though the thorough characterization of *Bacillus* provides a great starting point, little effort has been put into metabolic engineering strategies to increase heterologous production of natural products in *B. subtilis*. One successful example is the heterologous biosynthesis of the polyketide 6dEB. In this study, the *prpBD* operon responsible for the conversion of propionyl-CoA to succinate was deleted, resulting in a 75% increase in 6dEB production, but only in medium supplemented with propionate.[19]

Currently, the majority of metabolic engineering studies have focused on enhancing native surfactin production in *B. subtilis*. Several recent studies have successfully engineered the fatty acid biosynthetic pathway, resulting in increased production levels of this lipopeptide natural product. Strategies, such as increasing the pool of CoA-linked precursors by inhibiting their utilization for branched-chain fatty acid biosynthesis, overexpression of the rate-limiting enzyme in fatty acid biosynthesis to increase malonyl-ACP formation, and relieving the feedback inhibition on fatty acid synthesis by inhibiting degradation steps have all been shown to be effective.[197,208,209] However, it is important to consider the potential impact on the production profile of different surfactin variants from the same BGC, as metabolic adaptations can shift the relative production levels.[209] Similar results have been reported for fengycin biosynthesis.[198] In another elegant study, CRISPRi was used to investigate the influence of amino acid metabolism on surfactin biosynthesis, allowing the identification of several genes that could be targeted for improved yields.[210]

4.1.1.7 Terpenoid biosynthesis. Regarding terpenoids, different genes can be overexpressed or downregulated, depending on the desired final product, as they are all produced from the same precursor and pathway. General engineering strategies include enhancing the MEP pathway through enzyme overexpression, attenuating the branch pathways, (down)regulating the TCA cycle, and engineering specific terpene synthases.[144]

For example, researchers have used metabolic engineering to enhance carotenoid production in *B. subtilis* by manipulating various biosynthetic genes, such as *crtM*, *crtN*, *dxs*, *ispD*, *ispF*, *ispH*, *ispC*, *ispE*, *ispG* and *ispA*. Overexpression of certain genes, such as *dxs* or *dxr*, has been found to increase carotenoid production. Additionally, by modifying the growth medium conditions and supplementing it with increased levels of salt, hydrogen peroxide and heat, the production of isoprene, the precursor to carotenoids, can also be improved.[14]

Amorphadiene, the precursor to the antimalarial drug artemisinin, can be produced in *B. subtilis* by introducing the amorphadiene biosynthetic pathway. This involves co-expression of the amorphadiene synthase gene (*ads*) with other genes involved in the MEP pathway, such as *dxs* and *idi*. Increasing the expression of *ads*, *ispA* and providing additional precursors through the MEP pathway has been shown to further enhance amorphadiene production.[14]

Similarly, taxadiene, the precursor to the anticancer drug paclitaxel, can be produced in *B. subtilis* by combining the expression of the taxadiene synthase gene (*txs*) with the regulated overexpression of the MEP pathway, including the FDP synthase gene *ispA*. Finetuning the expression of MEP pathway genes has been demonstrated to improve taxadiene production.[14]

The vitamin K2 menaquinone-7 (MK-7) has been synthesized in *B. subtilis* through modular pathway engineering. To boost MK-7 production, several approaches have been employed, including overexpression of genes involved in the MK-7 pathway (*menA*) and the MEP pathway (*dxs*, *dxr*/*ispC*, *yacM*/*ispD* and *yacN*/*ispF* together with *menA*), along with enhancement of glycerol metabolism by overexpressing *glpD* and downregulating *dhhB*. The choice of integration sites for overexpressing MEP pathway genes, with *menA*, *dxs* and *dxr* inserted into *yxlA, yjoB* and *ydeO*, respectively, has also been shown to impact MK-7 production. Additionally, dynamically balancing

cell growth *via* the Phr60-Rap60-Spo0A quorum-sensing molecular switch has further contributed to enhanced MK-7 production.[14]

4.1.2 Heterologous production in a genome-reduced background. The genome of *B. subtilis* was among the first to be fully sequenced, comprising over 4.2 Mbps of genetic information.[211] A significant part of the genome is devoted to natural product biosynthesis by native BGCs. Several of these secondary metabolites, such as surfactin and fengycin, have antimicrobial activity, which can complicate functional screenings of heterologous expression strains. Moreover, native secondary metabolism requires a significant amount of energy, along with transcriptional and metabolic resources. To circumvent these issues, researchers have developed genome-reduced *B. subtilis* strains. Surprisingly, in functional heterologous expression systems for compounds like 6dEB and enniatin, the deletion of native BGCs from the genome has been reported to have opposite effects on the yields of the heterologously produced compound.[19,69] It is worth noting the particular case of the surfactin BGC, as it also contains the *comS* gene, a natural competence regulator gene located within the *srfAB* coding sequence. To avoid the loss of competence capacity, a careful disruption of this BGC is imperative.

Genome reduction is a valuable strategy to facilitate bioactivity-guided screenings. Through the use of the markerless *manP* counter-selection system, a reduction of the *B. subtilis* genome to 3.6 Mbps has been achieved.[40] This reduction involved the deletion of various genetic elements, including antibiotic-producing BGCs (the sublancin 168, subtilosin, plipastatin, surfactin, bacilysin, bacilysocin, 3,3′-neotrehalosadiamine pathways and a cryptic PKS cluster), phages and cryptic prophages, ICEBs1, sporulation delaying protein toxin genes *sdpABC* and *sdpRI*, sigma factors σ^E and σ^F, and genes like *spoGA* and *bpr.* The resulting strain, named IIG-*Bs*20-4, did not show any growth defects compared to *B. subtilis* 168. In follow-up research, surfactin production in this strain was assessed.[75] Interestingly, in terms of growth rate and glucose to biomass conversion efficiency, the genome-reduced strain outperformed the reference JABs24 strain, while the overall surfactin yield and surfactin production per unit of biomass, unfortunately, lagged behind. Nevertheless, such clean-background strains remain valuable for characterizing cryptic BGCs and simplifying downstream processing.

An additional genome reduction of 36% has been achieved in the mini*Bacillus* PG10 strain.[212] This strain has demonstrated the ability to successfully produce lanthipeptides heterologously. Notably, the absence of extracellular proteases in this strain leaves these RiPPs in an inactive state by inhibiting leader peptide processing. This provides an advantage by preventing toxicity to the host without the need for resistance or immunity genes. On the other hand, for the production of unknown RiPPs, additional maturation proteases will be necessary to obtain and identify the final bioactive peptide.[54] These examples highlight the significant potential of genome-reduced strains in simplifying the screening and production of heterologous natural products. However, there are currently limited studies that have used such strains compared to *Streptomyces* hosts with clean backgrounds.[72,213,214]

4.2 BGC refactoring elements

Standardized and well-characterized genetic building blocks play a crucial role in simplifying and finetuning heterologous BGC expression. Examples of such refactoring elements include promoters, terminators, RBSs, and protein tags (Table 3). It is important to note that these elements have organism-specific requirements, such as GC content, regulatory signals, and codon preference.[37,38] In 2013, Radeck *et al.* laid the initial groundwork for establishing a repository of readily useable genetic elements specifically tailored for *Bacillus* species by developing the *Bacillus* BioBrick Box,[38] which later underwent further updates and refinements.[37] In addition to the refactoring elements in this BioBrick Box, this section will also explore other genetic components that have been used to optimize heterologous expression in *Bacillus* spp.

4.2.1 Promoters. Promoters enable the finetuning of heterologous expression levels. In the original *Bacillus* BioBrick Box, six promoters were included, spanning a range of more than three orders of magnitude in terms of promoter strength. Among the constitutive promoters in the original Box are the Anderson promoters P_{J23101}, P_{liaG}, P_{lepA}, and P_{veg}, ranked by increasing strength.[38] Apart from these, a variety of other constitutive promoters have been used for heterologous expression of BGCs in *B. subtilis*. For example, to optimize the expression of the microcin J25 and microcin Y BGC, P_{veg}, a native promoter from *B. subtilis*, and P_{bsp} from the *B. subtilis* bacteriophage SPO1 were tested. Both of these promoters supported higher production levels when compared to the native microcin J25 promoter. Additionally, P_{43} and a derivative with a modification in the −35 region, called P_{43p}, were evaluated.[88] P_{43} is a widely used promoter often used as a reference to evaluate the strength of newly identified promoters.[215] The P_{repU} promoter, originating from the replication gene *repU* of the staphylococcal pUB110 plasmid, has also been included in various heterologous expression constructs, including those used for enhancing the yield of iturin A,[216] mycosubtilin,[217] and surfactin.[218] In the case of mycosubtilin, the use of this promoter even led to the discovery of a new mycosubtilin congener with novel bioactivity.[217] Increased surfactin yields have also been achieved using the P_{veg} promoter. However, the use of constitutive promoters for yield enhancement did not work equally well for all strains tested in this study. While surfactin production was increased in the minor producer strain *B. subtilis* 3A38, it was decreased in *B. subtilis* DSM 10^T, the major producer strain.[219] In another study, different constitutive promoters, namely P_{hpaII}, P_{amy}, P_{vgb}, P_{srfA} and P_{sig}, were integrated in the *B. amyloliquefaciens* genome to overexpress the native inulin hydrolase gene *cscA* to boost poly-γ-glutamic acid production.[95] Other constitutive promoters, such as $PA2_{cup}$ and $PC2_{up}$, have been used to enhance the production of levan by optimizing the expression of regulatory elements.[96]

Inducible promoters can be used to generate a two-phase system, involving bacterial growth followed by the addition of

Table 3 Promoters, tags and reporter genes for *Bacillus* species

Constitutive promoters	Strength	Ref.
P_{J23101}	Weak	38
P_{liaG}	Intermediate	38
P_{lepA}	Strong	38
P_{veg}	Very strong	38, 88 and 219
P_{bsp}	Not reported	88
P_{43}	Not reported	88, 227 and 228
P_{43p}	Not reported	88
P_{repU}	Strong	216–218
P_{hpaII}	Strong	95
P_{amy}	Intermediate	95
P_{srfA}	Weak	95 and 229
P_{sig}	Not reported	95
P_{vgb}	Not reported	95
$PA2_{cup}$	Strong	96
$PC2_{up}$	Strong	96
P_{penP}	Not reported	230
P_{BH4}	Three times stronger than P_{srfA}	229
P_{ylbP}	Not reported	229
Inducible promoters	**Inducer**	**Ref.**
P_{liaI}	Bacitracin	38
P_{xylA}	Xylose	38, 54, 95, 96, 129, 228, 229 and 231
P_{grac}	IPTG	95 and 96
P_{sacB}	Sucrose	96
P_{spank}	IPTG	54
$P_{spank\text{-}hy}$	IPTG	54 and 230
P_{spac}	IPTG	222 and 232
$P_{spac\text{-}hy}$	IPTG	122
P_{acoA}	Acetoin	69 and 220
P_{glv}	Maltose	223 and 224
opuAA	Salt	225
P_{spaS}	Subtilin	222
$P_{spaS\text{-}mut}$	Subtilin	222
P_{N25}	IPTG	64
P_{qdoI}	Flavonoid	233
P_{T7lac}	IPTG	230
P2	45 °C	226
P7	45 °C	226
Phase-dependent auto-inducible promoters	**Phase of induction**	**Ref.**
P_{abrB}	Exponential	234
P_{valS}	Exponential	234
P_{hag}[234]	Exponential	234
P_{asd}	Middle-log and early stationary	234
P_{spoVG}	Middle-log and early stationary	234
P_{lytR}	Lag–log and stationary	88 and 234
P_{gsiB}	Lag–log and stationary	95 and 234
P_{mmgA}	Stationary	234
P_{sigW}	Stationary	234
P_{yafD}	Stationary	234

Table 3 (*Contd.*)

Epitope tags	Full name	Ref.
StrepII	Strep-Tactin-tag	38
His_{10}	10× His-tag	38
HA	Human influenza hemagglutinin-tag	38
cMyc	cMyc-tag	38
FLAG	FLAG-tag	38
His_6	6× His-tag	129 and 224
SUMO	Small ubiquitin-like modifier-tag	224
GST	Glutathione *S*-transferase-tag	129
ST	Strep-tag	129
Secretion tags		**Ref.**
SP_{yncM}		95 and 96
SP_{amyQ}		95
SP_{sacB}		95 and 96
SP_{sacC}		95
SP_{npr}		95
SP_{amyE}		96
SP_{bglS}		96
SP_{bpr}		96
SP_{nprB}		96
SP_{nprE}		96
SP_{wapA}		96
Reporter gene	**Reporter protein**	**Ref.**
lacZ	β-Galactosidase	38
luxABCDE	Luciferase	38
mCherry	Red fluorescent protein	37
mTagBFP	Blue fluorescent protein	37
eCFP	Cyan fluorescent protein	37
sfGFP	Green fluorescent protein	37
GFPmut1	Green fluorescent protein	37
mEYFP	Yellow fluorescent protein	37
SYFP2	Yellow fluorescent protein	37

an inducer to activate the production system.[220] The original *Bacillus* BioBrick Box includes two inducible promoters: P_{liaI} and P_{xylA}. P_{liaI} is induced by the antibiotic bacitracin, while P_{xylA} can be induced by adding xylose to the growth medium. For the optimal functioning of P_{xylA}, fructose has been suggested as the best carbon source.[38] The P_{xylA} promoter has been used in combination with the XylR repressor for heterologous production of the thiopeptide micrococcin P1.[129] Another study, aimed at enhancing poly-γ-glutamic acid, evaluated two inducible promoters for overexpression of the inulin hydrolase gene *cscA*: P_{xylA} and the IPTG-inducible promoter P_{grac}.[95] Both of these promoters were also tested alongside the sucrose-inducible promoter P_{sacB} to boost levansucrase expression for enhanced levan production, with P_{grac} yielding the best results.[96,221]

Furthermore, van Tilburg *et al.* established a heterologous expression system to generate various hybrid lanthipeptides by controlling the expression of the modification and export genes with P_{xylA} and regulating the expression of the precursor peptide genes with two IPTG-inducible promoters, P_{spank} and $P_{spank\text{-}hy}$.[54] The latter is the hyper version of P_{spank} and is six times stronger.[54] Another inducible promoter, $P_{spac\text{-}hy}$, was used for heterologous expression of various gut microbiota-derived NRPS clusters in *B. subtilis*.[122] This promoter is a derivative of the P_{spac} promoter, which combines elements from a *B. licheniformis* penicillinase promoter and the *E. coli lac* promoter.[222] P_{acoA}, which is repressed by glucose and induced by acetoin, has been used for the recombinant production of the fungal NRP enniatin. However, excessive levels of the acetoin inducer resulted in cell lysis.[69] In 2006, Ming-Ming *et al.* developed the maltose-inducible promoter P_{glv}, which is repressed in the presence of glucose.[223] This promoter has been successfully used in *B. subtilis* WB800N for recombinant production of T9W, a variant of the pig myeloid antimicrobial peptide-36 effective against *Pseudomonas aeruginosa*. Optimal production was achieved with the addition of 5% maltose as an inducer.[224] For a more cost-effective and straightforward induction, the salt-inducible promoter *opuAA* has been employed. This promoter is part of the natural stress-response system in *B. subtilis*. When tested by expressing a protease gene in *B. subtilis* WB800, a nine-fold increase in expression was observed upon induction with 4% NaCl compared to non-inducing conditions.[225] Some promoters can also be induced by a change in temperature. Examples include P_2 and P_7, which show higher expression levels at 45 °C compared to 37 °C in both *E. coli* and *B. subtilis*.[226]

To enable a more dynamic production of subtilin in *B. subtilis*, a system for subtilin-regulated gene expression (SURE) was established, analogous to the nisin-controlled gene expression (NICE) system in *Lactococcus lactis*. The promoter of the subtilin BGC (P_{spaS}) is regulated by the two-component system SpaKR, which is autoactivated by subtilin. Based on this principle, vectors were designed for subtilin-inducible heterologous expression: one vector for integration of the *spaKR* regulatory system into *amyE*, along with expression vectors containing the P_{spaS} promoter and a mutant derivative $P_{spaS\text{-}mut}$. The P_{spaS} promoter was shown to be more strictly controlled, while the $P_{spaS\text{-}mut}$ resulted in higher expression levels. The SURE system has the potential to be used for the heterologous expression of any gene of interest.[222]

Certain promoters can be induced based on the growth phase of the bacteria. Such phase-dependent auto-inducible promoters are classified in four categories depending on the phase during which they are activated: class I (exponential phase), class II (middle-log and early stationary phase), class III (lag–log and stationary phase), and class IV (stationary phase). In one study, 114 endogenous *B. subtilis* promoters were selected from the DBTBS database and experimentally characterized to classify them into these four classes. Class I promoters include P_{abrB}, P_{valS}, and P_{hag}. Class II is represented by P_{asd}, with P_{spoVG} showing the highest activity among this class. Class III promoters exhibit linearly increasing activity as cells grow, with P_{lytR} being the promoter with the highest activity in this class.[234] This promoter has been used for the successful heterologous production of microcin J25 and Y.[88] P_{gsiB}[234] is another representative of class III and has been used to overproduce inulin hydrolase for enhanced poly-γ-glutamic acid yield.[95] Examples of class IV promoters include P_{mmgA}, P_{sigW}, and P_{yqfD}. Some of these promoters also exhibit pH and temperature dependencies.[234]

The promoters listed here have been successfully used for the heterologous expression of natural product BGCs. For a more general overview of effective promoters in *Bacillus*, the reader is referred to an excellent review by de Souza *et al.*[215]

4.2.2 Protein tags and signal peptides. On a protein level, tags can serve as valuable tools to facilitate purification and improve secretion. The choice of whether to add a tag to the N- or C-terminus depends on the specific protein being studied. The original *Bacillus* BioBrick Box contains five such epitope tags: the StrepII-, His_{10}-, HA-, cMyc-, and FLAG-tag.[38]

For the purification of the *P. aeruginosa* antimicrobial peptide T9W, a His_6-tag was appended to the N-terminus of the peptide. Additionally, a SUMO-tag was inserted between the His_6-tag and T9W to enable efficient removal of the His_6-tag. This SUMO-tag also helps to improve the stability and expression level of recombinant proteins.[224]

To increase the production of poly-γ-glutamic acid, inulinases involved in the production process have to be exported *via* a peptide-dependent signalling pathway. Therefore, five signal peptides were assessed: SP_{yncM}, SP_{amyQ}, SP_{sacB}, $SP_{sacC,}$ and SP_{npr}, with SP_{npr} showing the best results.[95] A similar strategy has been employed to enhance levan production by adding signal peptides to the N-terminus of levansucrase. Various signal peptides, including SP_{amyE}, SP_{bglS}, SP_{bpr}, SP_{nprB}, $SP_{nprE,}$ and SP_{wapA} were used, in addition to SP_{sacB} and SP_{yncM}.[96] For the purification of the thiopeptide micrococcin P1, a His_6- or glutathione *S*-transferase (GST)-tag was added to the N-terminus of the compound. Additionally, Strep-tags were attached to either the N- or C-termini of the modifying enzymes.[129]

4.2.3 Reporter proteins. To measure expression levels and quantify promoter activities, various reporter genes can be employed. In the original BioBrick Box, only two reporter genes were included: *lacZ* for colorimetric measurements and the *luxABCDE* operon for luciferase-based assays.[38] Meanwhile, seven fluorescent proteins spanning the entire visible light spectrum have been added, with the corresponding genes codon optimized for *B. subtilis*. These reporters offer the advantage of being able to be translationally fused to a protein of interest. Among these fluorescent proteins, red mCherry exhibits a particularly high signal-over-background ratio. The others are blue mTagBFP, cyan eCFP, green sfGFP and GFPmut1, and yellow mEYFP and SYFP2.[37]

4.2.4 Vectors. Refactored BGCs and other genetic circuits and elements are typically integrated in an expression vector (Table 4). These are often designed as shuttle vectors to enable cloning in *E. coli*, followed by expression in *B. subtilis*.[37,38,220,235] All vectors within the original BioBrick Box are equipped with an *E. coli*-compatible origin of replication (ori) and an ampicillin resistance marker (*bla*). For transformation of *B. subtilis*,

Table 4 Vectors for heterologous expression in *Bacillus* species. The type column indicates whether and at which location the vector integrates into the genome or whether it stays present as a replicative plasmid

Vector	Type	Resistance	Additional information	Ref.
pDG1662	Integrates in *amyE*	Chloramphenicol		235
pDG1730	Integrates in *amyE*	Spectinomycin		235
pDG1664	Integrates in *thrC*	Macrolide-lincosamide-streptogramin B		235
pDG1731	Integrates in *thrC*	Spectinomycin		235
pBS1C	Integrates in *amyE*	Ampicillin and chloramphenicol		38
pBS1E	Integrates in *amyE*	Ampicillin and macrolide-lincosamide-streptogramin B		37
pBS1K	Integrates in *amyE*	Ampicillin and kanamycin		37
pBS2E	Integrates in *lacA*	Ampicillin and macrolide-lincosamide-streptogramin B		38
pBS4S	Integrates in *thrC*	Ampicillin and spectinomycin		38
pSD193	Integrates in *srfA*	Ampicillin and kanamycin		64
pSD270	Integrates in *srfA*	Ampicillin and kanamycin	C-Terminal His_6-tag	64
pKE151	Integrates in *srfA*	Ampicillin and kanamycin		64
pKE170	Integrates in *srfA*	Ampicillin and kanamycin	C-Terminal His_6-tag	64
pBS0E	Replicative vector with ori1030	Ampicillin and macrolide-lincosamide-streptogramin B		37
pBS1C*lacZ*	Integrates in *amyE*	Ampicillin and chloramphenicol	β-Galactosidase reporter	38
pBS3C*lux*	Integrates in *sacA*	Ampicillin and chloramphenicol	Luciferase reporter	38
pBS3E*lux*	Integrates in *sacA*	Ampicillin and macrolide-lincosamide-streptogramin B	Luciferase reporter	37
pBS3K*lux*	Integrates in *sacA*	Ampicillin and kanamycin	Luciferase reporter	37
pBS3E*catlux*	Integrates in *sacA*	Ampicillin and macrolide-lincosamide-streptogramin B	Luciferase reporter combined with chloramphenicol resistance	37
pBS3K*catlux*	Integrates in *sacA*	Ampicillin and kanamycin	Luciferase reporter combined with chloramphenicol resistance	37
pBS1C*αlacZ*	Integrates in *amyE*	Ampicillin and chloramphenicol	LacZ-α part of β-galactosidase reporter, for screening of RBS libraries	37
pBS3C*αlux*	Integrates in *sacA*	Ampicillin and chloramphenicol	LacZ-α part of β-galactosidase reporter, for screening of RBS libraries	37
pBS2EP_{xylA}	Integrates in *lacA*	Ampicillin and macrolide-lincosamide-streptogramin B	Xylose-inducible	37
pBS2EP_{liaI}	Integrates in lacA	Ampicillin and macrolide-lincosamide-streptogramin B	Bacitracin-inducible	37
pBS2EXylRP_{xylA}	Integrates in *lacA*	Ampicillin and macrolide-lincosamide-streptogramin B	Xylose-inducible, XylR repressor present	37
pBS0EP_{liaI}	Replicative vector with ori1030	Ampicillin and macrolide-lincosamide-streptogramin B	Bacitracin-inducible	37
pBS0EXylRP_{xylA}	Replicative vector with ori1030	Ampicillin and macrolide-lincosamide-streptogramin B	Xylose-inducible, XylR repressor present	37 and 136
pMSE3	Replicative vector with pAMβ1 ori	Kanamycin		69 and 220
pDR111	Integrates in *amyE*	Ampicillin and spectinomycin	IPTG-inducible	136

the vectors are linearized *via* a unique *ScaI* restriction site located within the *bla* gene.[38] Due to the challenges often associated with heterologous NRP production in *E. coli*, a shuttle vector system has been developed to enable parallel gene expression in both *E. coli* and *B. subtilis*. Four vectors have been constructed for this purpose: pSD193, pSD270, pKE151 and pKE170. All these vectors contain an IPTG-inducible T5 P_{N25} phage promoter for control of gene expression in both host organisms. The integration in the *B. subtilis* genome takes place at the deleted *srfA* locus. All vectors contain an *E. coli* ColE1 ori and both a kanamycin and ampicillin resistance marker. They differ in their restriction enzyme recognition sites in the multiple cloning site (MCS) and in the presence of a C-terminal His_6-tag.[64]

It is common practice to integrate heterologous BGCs into the genome of *B. subtilis*. Examples of vectors suitable for integration in the *amyE* locus are pDG1662 and pDG1730, which confer chloramphenicol and spectinomycin resistance, respectively. For integration into the *thrC* locus, pDG1664 and pDG1731, which provide MLS (macrolide-lincosamide-streptogramin B) and spectinomycin resistance, respectively, can be used.[235] The original *Bacillus* BioBrick Box consists of five BioBrick-compatible integrative *B. subtilis* vectors, three of which are equipped with compatible resistance markers and integration sites. These vectors are derived from the *B. subtilis* vectors pDG1662, pAX01 and pDG1731, which were modified to be BioBrick-compatible, resulting in pBS1C, pBS2E and pBS4S, respectively. They can be used to introduce any construct of interest into the *amyE*, *lacA*, and *thrC* loci, respectively. The integrative part of these vectors consists of flanking homology regions, a chloramphenicol, MLS or spectinomycin resistance cassette for selection in *Bacillus* and a MCS containing type II restriction enzyme recognition sites. Situated between these recognition sites in the MCS is an *rfp*-cassette. During the cloning process, this cassette is replaced with the desired insert, resulting in a white colour instead of a red when screening for positive hits in *E. coli*.[38] In the updated version of the BioBrick Box, new pBS1C derivatives were included, in which the chloramphenicol resistance gene was replaced with either an MLS or kanamycin resistance cassette.[37]

The original *Bacillus* BioBrick Box also includes two reporter vectors derived from pAC6 and pAH328. One contains the *lacZ* reporter gene, known as pBS1ClacZ, while the other carries the *luxABCDE* operon, known as pBS3Clux. They are designed for integration into the *amyE* and *sacA* loci, respectively, and they both harbour a selection marker conferring chloramphenicol resistance.[38] In the updated version of the BioBrick Box, the chloramphenicol resistance gene of pBS3C*lux* was replaced with either an MLS or kanamycin resistance cassette, resulting in the derivative vectors pBS3E*lux* and pBS3K*lux*, respectively. These vectors were further equipped with a chloramphenicol resistance gene as co-selection marker in front of the *luxABCDE* operon. This allows for a more robust screening of promoter libraries and facilitates the analysis of promoter strengths. Moreover, reporter vectors designed to screen RBS libraries were generated as derivatives of pBS1C*lacZ* and pBS3C*lux*. For this purpose, a *lacZα* fragment was inserted downstream of the MCS. This arrangement allows for red/blue/white screening in *E. coli* upon introduction of a promoter in the MCS and an RBS at the position of *lacZα*. Five different RBS variants, differing by a few point mutations from the consensus sequence AA AGG were screened using this system.[37]

As an alternative to integrative vectors, replicative vectors that do not integrate themselves in the genome can be used for heterologous BGC expression in *Bacillus*. The updated *Bacillus* BioBrick Box was expanded to include such a replicative vector, known as pBS0E, which is derived from the medium-copy vector pGP380. Also here, an *rfp*-cassette was introduced into the MCS to facilitate red-white screening during cloning in *E. coli*.[37] Another example is pMSE3, a replicative shuttle vector compatible with both *E. coli* and *B. subtilis*.[220] The use of this vector has resulted in higher yields during recombinant enniatin production compared to integration in the genome. This replicative plasmid led to the presence of about 200 copies of the enniatin biosynthetic genes in the *Bacillus* strain.[69]

To ensure reproducibility, it is important to have vectors that are already equipped with promoters, so that genes of interest can be introduced directly in the MCS for expression. Therefore, the BioBrick Box was updated to contain redesigned versions of pBS2E and pBS0E that carry an inducible promoter, such as P_{liaI}, P_{xylA}, or a combination of P_{xylA} with its repressor *xylR*, upstream of the MCS.[37] This latter vector was used in combination with the integrative vector pDR111 to overexpress the paenithopeptin precursor peptide gene from the replicative vector, while integrating one copy of the post-translational modification genes for this lanthipeptide into the genome.[136] An exhaustive list of all vectors that are available for heterologous BGC expression in *Bacillus* can be found on *Subti*Wiki.[17,55]

4.2.5 Library-based refactoring elements. To further enhance the tunability range and expand the selection of refactoring elements, large libraries of these components have been screened.[215] This effort has resulted in the development of a comprehensive toolbox containing a diverse array of regulatory elements to control recombinant production in *B. subtilis* over many orders of magnitude at the level of transcription, translation, and protein degradation. This library was created by integrating synthetic constitutive promoters, RBS sequences, and SsrA degradation tags along with a *gfp* reporter gene, into the *amyE* locus of *B. subtilis*. The synthetic constructs were created by amplifying the GFP gene using primers that had degenerated regulatory regions at strategic positions. For the promoter library, three strong promoters P_{veg}, P_{serA}, and P_{ymdA} were randomized at the −10 and/or the −35 box, or in between these regions. For the RBS library, three RBSs from highly and constitutively expressed genes were selected. A library was then constructed by introducing mutations into the six nucleotides upstream of the start codon. Finally, a degradation tag library was generated by modifying the final three residues of the SsrA tag, which can be added to the C-terminus of a protein to mark it for proteolysis by cellular proteases. The resulting library was screened by measuring GFP fluorescence in *B. subtilis*. This process enabled the precise tuning of GFP production from nanomolar to millimolar concentrations.[236] A particular

advantage of using synthetic promoters in this context is that they are less prone to cell stress and high metabolic demands.[215]

In another study, a system was devised in which a cryptic promoter was activated by a spontaneous mutation to enable inducer-free activation of gene expression. The bacteria harbouring this mutation were selectively enriched due to a growth advantage linked to glutamate homeostasis. To simplify the detection of active mutations, a *gfp* gene was integrated into the construct.[237]

Han *et al.* developed a tool in *B. subtilis* named 'Stepwise Evolution Targeting the Spacer region of Core Promoter' (SETarSCoP).[229] This method offers an effective way for evolving the strength of bacterial promoters. In a first step, the spacer sequences between the −35 and −10 box were compared in strong and weak native promoters of *B. subtilis* 168. This analysis revealed a conserved region of seven bp upstream of the −10 box. In a second step, this insight was applied to P_{srfA}, a constitutive promoter with higher strength than P_{43}. *Via* a two-step, random mutagenesis process, the mutant promoter P_{BH4} was created, which exhibits threefold higher strength compared to P_{srfA}. To further demonstrate its applicability, the same approach was used to improve the strength of the constitutive promoter P_{ylbP} and the xylose-inducible promoter P_{xylA}.[229]

4.2.6 T7 RNA polymerase-based expression systems. A bacteriophage T7-based expression system is one of the most widely used methods for heterologous expression in *E. coli*. The system has been adapted for use in *B. subtilis* and its efficacy evaluated through heterologous expression of both cytosolic and secretory proteins. Several alternatives based on the T7 RNA polymerase system have been developed, which can be categorized into two groups.[227,228,230–233]

In the first group, the gene of interest is expressed under the control of a T7 promoter on a replicative plasmid, while the T7 RNA polymerase is integrated into the chromosome.[227,228,231,233] Conrad *et al.* pioneered the development of a T7 RNA polymerase-dependent system for heterologous expression in *Bacillus*.[231] In this system, the T7 RNA polymerase gene *rpoT7* was integrated into the *amyE* gene of the *B. subtilis* chromosome, under the regulation of the XylR-P_{xylA} xylose-inducible system. In addition, a replicative plasmid was constructed for inserting the gene of interest between a T7 promoter and terminator. However, rifampicin had to be added to the medium to repress the host RNA polymerase. Moreover, the presence of the T7 RNA polymerase appeared to affect the processing and/or the secretion of the recombinant proteins.[231] More recently, an analogous system was implemented in *B. subtilis* 164S, which is derived from the industrial strain *B. subtilis* ATCC 6051a. The same xylose-inducible regulatory cassette was used but integrated in the *aprE* gene instead of *amyE*.[228] Consequently, the protease encoded by *aprE* was no longer functional.[227] When comparing GFP production in this system to GFP production driven by the constitutive P_{43} promoter, a ten-fold increase in yield was observed. This system was also employed to recombinantly produce an α-L-arabinofuranosidase enzyme, which degrades arabinoxylan polymers to release D-xylose, making the system self-inducible.[228] However, a limitation of this system is that the inducer, xylose, is continuously degraded by the host.[227,228] To circumvent this issue, Ye *et al.* developed an IPTG-inducible system in *B. subtilis*.[227] This strain was equipped with a xylose-inducible *comK* gene to facilitate biotransformation. It also featured deletions in protease genes like *aprE* and *nprE*. Additionally, genes related to sporulation (*spoIIAC*) and surfactin synthesis (*srfAC*), which can cause foam formation during fermentation, were inactivated to ensure optimal recombinant production and high cell densities during fermentation. The T7 RNA polymerase gene was integrated into the *amyE* locus on the *B. subtilis* SCK6 chromosome, under the control of the constitutive P_{43} promoter. To express genes of interest, a plasmid, named pHT7, was constructed, featuring a hybrid T7-lac promoter, a T7 terminator, and a *B. subtilis* RBS. Genes can be efficiently introduced in this plasmid using prolonged overlap extension polymerase chain reaction (POE-PCR), followed directly by transformation. The effectiveness of this engineered system was demonstrated through the expression of various fluorescent proteins and enzymes.[227]

Recently, a flavonoid-inducible system was developed. In this system, the T7 RNA polymerase gene was integrated into the *amyE* gene of the *B. subtilis* 168 chromosome under the control of P_{qdoI}. This promoter contains two boxes to which the repressors LmrA and QdoR bind in the absence of flavonoids, such as quercetin and fisetin. The gene of interest was expressed on a multicopy plasmid under the control of the T7 promoter. The functionality of the system was tested by heterologous production of EGFP. No leaky expression was observed and induction was dependent on the specific flavonoid used. Additionally, an engineered version of P_{qdoI} was implemented, which exhibited increased, but also more leaky expression.[233]

In the second group of T7 RNA polymerase-dependent systems, the gene of interest is integrated into the chromosome to enhance genetic stability.[230,232] In one such system, the T7 RNA polymerase gene, *T7 gene 1*, was placed under the control of P_{spac}, and integrated into the *wprA* gene of *B. subtilis* DB428, which encodes a cell wall-associated protease. The T7 promoter and the gene of interest were introduced in the *mpr* gene, which encodes an extracellular protease. By strategically targeting these genes for integration, six proteases were eliminated from the producer strain, which had a positive effect on recombinant production.[232] In an IPTG-inducible system established in *B. subtilis* PY79, the gene of interest was placed under the control of P_{T7lac}, a chimeric promoter controlled by both the T7 RNA polymerase and the presence of IPTG. This design significantly reduced the leakiness of the system. The T7 RNA polymerase gene was driven by a $P_{hy\text{-}spank}$ promoter, while the LacI repressor was constitutively expressed from a P_{penP} promoter.[230] This system showed a dynamic range of over 10 000, surpassing the capabilities of the previously described systems,[231,232] which had dynamic ranges up to 50.[230]

4.3 BGC cloning strategies and engineering tools

Despite the increasing number of BGCs identified through genome sequencing, only a small fraction has been experimentally characterized and linked to a metabolic product.[238] Silent BGCs, which are not expressed under standard laboratory conditions, hold significant potential for the discovery of novel drugs.[239–242] However, the large-scale activation of silent BGCs poses challenges

due to the lack of universal strategies and enabling technologies.[242] Two main strategies are employed to tackle this problem: native host-based and heterologous host-based approaches. Native host-based methods maintain BGC integrity but rely on strain-specific genetic tools, making them unsuitable for genetically intractable or uncultivated strains. Heterologous host-based methods offer more flexibility, allowing for the cloning, heterologous expression, and functional characterization of diverse types of natural product BGCs, including those identified through metagenomics. Cloning large BGCs remains a limiting step, but various methods, including library construction, PCR-based assembly, and direct cloning, have been developed (Fig. 2).[243,244] Direct cloning offers advantages by eliminating the need for library construction and minimizing random mutations. It holds promise for large-scale discovery of bioactive natural products. In this section, we will focus on methods that are specifically developed for heterologous expression of natural product BGCs in *Bacillus*. A method that has not yet been widely used for BGC cloning, but is making fast progress, is *de novo* DNA synthesis. While fragments of 5–7 kb can now be ordered routinely for less than $0.10/bp, some manufacturers offer even longer fragments of 50 kb and more. However, at a current price of $0.50–1/bp and lead times of several months, direct BGC synthesis remains out of reach for most researchers in the natural product discovery field.[245] Conversely, so-called biofoundries, highly automated facilities for synthetic biology that largely rely on synthetic DNA, are becoming increasingly popular and accessible. Recent efforts to harness their capabilities for natural product discovery have yielded promising results.[246] It can therefore be anticipated that in the future, when prices drop, natural product discovery will be able to benefit from complete, *de novo* BGC synthesis combined with bottom-up refactoring techniques.[242]

4.3.1 Library-based techniques. In the early stages of microbial BGC cloning, library-based methods played a crucial role, providing a relatively straightforward way of obtaining physical access to BGCs. These approaches involve the creation of a clone library of random genomic DNA fragments in *E. coli*. This is particularly useful when complete genome sequences are not available, or in the case of environmental DNA.[247] Cosmid[247,248] and fosmid[247,249,250] libraries are mostly used to clone BGCs from cultured and uncultured organisms, as well as metagenomic samples, with cosmid libraries also being used for soil metagenomic DNA.[251] However, BGCs often need to be assembled from multiple clones, which is a significant disadvantage and requires substantial screening efforts.[247,251] Moreover, these methods were initially limited to fragments of around 40 kb in size, restricting their applicability to smaller BGCs. Larger DNA inserts (>100 kb) have been achieved through the use of library vectors, such as bacterial artificial chromosomes (BACs) and P1 artificial chromosomes (PACs), allowing for more comprehensive characterization of BGCs.[247] Furthermore, a self-replicating fungal artificial chromosome (FAC) was developed for *Aspergillus*, enabling direct transfer and heterologous expression of FAC libraries.[252]

A major advantage of clone libraries is that, once constructed, they can be screened numerous times in search of diverse BGCs of interest. However, despite improvements in the library construction process, the identification and recovery of clones carrying target BGCs from millions of unrelated clones remains a major bottleneck. This typically involves iterative rounds of sequential serial dilution and PCR, a highly laborious task.[253] In this context, it is interesting to highlight a recently developed platform for plasmid library enrichment that combines microfluidics and droplet PCR.[254] Using this platform, each clone in a library can be individually interrogated for

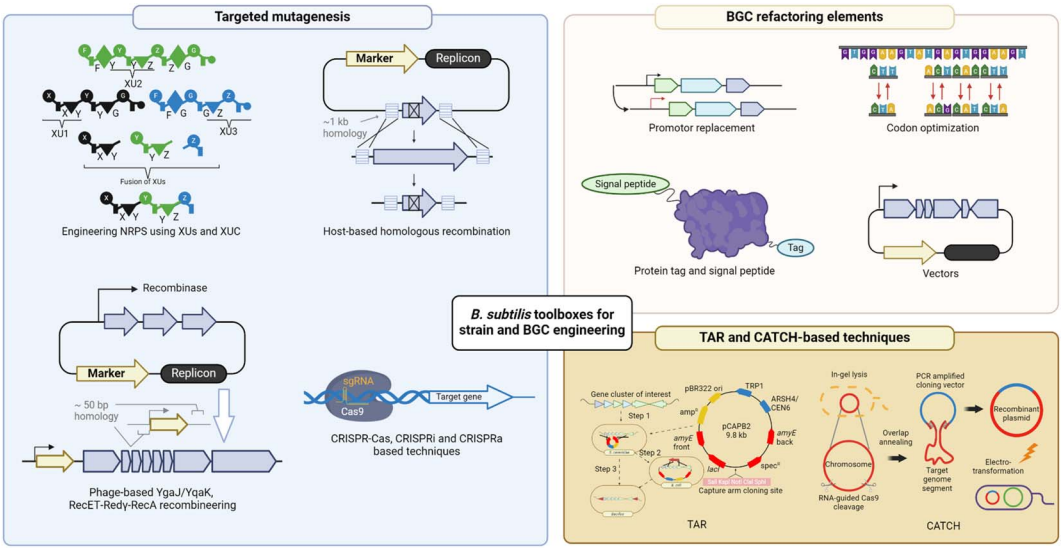


Fig. 2 Overview of *Bacillus* toolboxes for strain and BGC engineering discussed in this review. Different targeted mutagenesis approaches for strain engineering are depicted on the left panel, together with the exchange units (XUs) approach for specific NRPS engineering. Genetic elements for BGC refactoring described in this review are grouped on the right upper panel. Below, two techniques for the direct capture of BGCs are depicted; transformation-associated recombination (TAR) and Cas9-assisted targeting of chromosome segments (CATCH). Figure was created with Biorender, and adapted from ref. 50, 243, 244, 268 and 292.

the presence of a target insert at a throughput of thousands per second, yielding a pool of purified and sequence-enriched plasmid DNA. While only demonstrated as a means to achieve targeted sequencing of rare clones, it may also be possible to recover and amplify the enriched plasmids by transformation, offering an attractive and cost-effective route to rapidly deliver BGCs into the hands of researchers.[254]

A unique and undervalued feature of PCR-based screening of metagenomic libraries is the capability to isolate completely novel BGCs, even if the PCR primers are designed based on known genetic elements. This has most convincingly and extensively been demonstrated by Sean Brady and colleagues, who have used degenerate primers and amplicon sequencing to identify previously unknown BGCs that assemble structurally distinct natural products with novel modes of action.[253,255] A well-known example is the discovery of the malacidins, relatively common calcium-dependent antibiotics that nevertheless were not previously discovered by culture-dependent methods. This distinctive class of antibiotics was identified using primers that target highly conserved NRPS domains.[251] The potential of rationally designed PCR assays, utilizing degenerate primers to target class-defining enzymes for novel BGC identification from metagenomic libraries, combined with a plasmid enrichment platform as described above for physical BGC isolation, and subsequent transfer into appropriate heterologous expression systems, offers exciting prospects for future drug discovery efforts.

So far, the use of library-based techniques for heterologous expression of BGCs in *Bacillus* has been relatively limited. Luo *et al.* constructed a fosmid library from *B. velezensis* 916 to isolate the BGC for the NRP locillomycin.[99] The library was introduced into *E. coli* using the lambda phage and PCR analysis identified the fosmid that contained the locillomycin BGC. Next, the fosmid was modified to enable the heterologous expression of the BGC in *B. velezensis* FZB42. This was done by introducing a spectinomycin resistance cassette and the IPTG-inducible P_{spac} promoter using λ Red recombination. The authors showed that locillomycin production was increased by more than 15-fold compared to the native *B. velezensis* 916 strain.[99] The same approach was also applied to produce the enniatin synthetase gene of *Fusarium oxysporum* in *B. subtilius*.[69]

4.3.2 Direct capture techniques. Targeted approaches for BGC cloning, such as assembly and direct cloning methods, have gained popularity due to the ever increasing availability of genome sequence information and vastly improved cloning techniques.[247] The *S. cerevisiae* transformation-associated recombination (TAR)-based method, is a technique developed in 1996 to selectively clone large consecutive DNA fragments from human genomic DNA.[256,257] It takes advantage of the high recombination capacity of homologous DNA molecules when introduced into yeast. In 2010, the TAR method was adapted to create a shuttle capture vector, named pTARa, designed for the direct cloning, maintenance, and manipulation of large BGCs in *S. cerevisiae*. This vector facilitates heterologous expression in *Streptomyces* hosts *via* integrative conjugation. The pTARa vector was successfully used to clone the colibactin BGC from *Citrobacter koseri* and to reassemble three complete BGCs from soil-derived environmental DNA cosmid libraries.[258]

In 2014, a new vector known as pCAP01 was developed for TAR cloning, which can exist in multiple copies in *E. coli*. It was used to clone a genomic region containing the taromycin BGC from *Saccharomonospora* sp. CNQ490. Although the initially cloned *tar* cluster was not efficiently expressed in *S. coelicolor* M1146, subsequent gene remodelling led to successful activation and production of taromycin A, a compound highly similar to but structurally distinct from daptomycin. The pCAP01 vector can be used to clone various types of natural product BGCs.[46] To improve the efficiency of TAR cloning, a counter-selectable marker gene, URA3, was introduced, resulting in pCAP03. This vector showed higher capture rates and reduced self-circularization. Additionally, a ready-to-use version of pCAP03 (RTU-pCAP03) was designed, eliminating the need for PCR in capture plasmid construction and thereby increasing capture efficiency.[73,259] In 2019, a new vector called pCL01 was developed to facilitate the capture of larger BGCs in a single-copy form. It also allows higher copy numbers of cloned BGCs within *E. coli* cells. This vector was used to capture the 5-oxomilbemycin BGC and subsequently enhance its production using genome engineering.[260] In the meantime, various derivative TAR cloning vectors have been developed to expand the range of heterologous hosts for natural product biosynthesis, including the pCAPB02 vector for *B. subtilis*[50,259] and the pCAP05 vector for broad-host-range expression in Gram-negative hosts.[261]

The pCAPB02 vector combines yeast elements from pCAP01, *E. coli* elements from pBR322, and a homologous recombination-based integration system for *B. subtilis* from pDR111. It can be selected in *E. coli* and *B. subtilis* using ampicillin and spectinomycin. The insertion site for the BGC is located between the 5′ and 3′ gene fragments of the conserved *B. subtilis amyE* gene, allowing for double-crossover recombination into the genomes of various *B. subtilis* strains.[259] So far, the vector has been used to successfully clone and express the amicoumacin BGC (PKS-NRPS hybrid, 47.4 kb) from *B. subtilis* 1779,[50] the plipastatin BGC (NRPS, 39 kb) from *B. amyloliquefaciens* HYM12 (ref. 123) and the iturin BGC (NRPS, 38 kb) from *B. amyloliquefaciens* HYM12.[262]

A further advancement was made by combining CRISPR/Cas9-mediated *in vitro* digestion of DNA with TAR cloning.[263] This method, reported by Lee, Larionov, and Kouprina, has led to a significant increase in the success rate of obtaining positive clones. By using CRISPR/Cas9, DNA can be precisely cut at specific locations, thereby enhancing the efficiency of TAR cloning.[263] While this approach has not yet been applied to TAR cloning of microbial BGCs for heterologous expression, it has the potential to be widely applicable to various direct cloning methods. It has already been used in combination with other methods, such as RecE-catalyzed linear–linear homologous recombination (LLHR) and Gibson assembly. It offers a promising avenue for improving the efficiency of TAR cloning and expanding its applications in the future.[247]

Another direct capture technique that has been optimized for heterologous expression of BGCs in *B. subtilis* is LLHR. In 2012, Fu *et al.* introduced the LLHR strategy, which relies on

homologous recombination between linear DNA molecules facilitated by RecE and RecT Rac prophage proteins.[264] The procedure involves designing PCR primers, generating a linear capture vector, digesting genomic DNA, and co-electrotransformation of an engineered *E. coli* strain GB05-dir, where RecET-Redγ-RecA are integrated in the genome under the control of a P_{BAD} promoter. This method has been successfully used to clone PKS-NRPS BGCs from *Photorhabdus luminescens*. However, the LLHR strategy showed limitations when cloning larger gene clusters due to self-circularization of the capture vector. To address this challenge, the authors combined LLHR with Redαβ-mediated linear plus circular homologous recombination (LCHR) in a two-step cloning approach, allowing them to clone the 52 kb *plu2670* gene cluster.[242,264] Another example of the successful use of LLHR is the cloning of the cryptic *nrs* BGC.[21] Heterologous expression in *B. subtilis* of this BGC led to the discovery of the bacillothiazoles, illustrating again the potential of *B. subtilis* as host for the mining of new compound classes. In the meantime, several groups have updated the method to accommodate specific needs or to generally improve the method. These updated versions include the ExoCET platform (Exonuclease Combined with RecET recombination)[265,266] and RedEx method.[242,267]

Liu *et al.* further adapted the LLHR method in 2016 to enable cloned gene clusters to be directly integrated into the genome of *B. subtilis* without the need for further modification.[20] They developed a simplified method in which a pair of primers with 70 bp homologous arms is used to generate a linear cloning vector through PCR. This vector, containing the desired gene cluster, can be directly integrated into the *B. subtilis* chromosome. The entire process can be completed within a week if the gene cluster is intact and lacks homologous regions with the host strain. Overall, there are three main advantages of this adapted LLHR method, compared to TAR cloning. Firstly, it relies on Red/ET recombineering in *E. coli*, which simplifies the process by using shorter homologous arms and eliminating several steps associated with the TAR method. Secondly, the use of a shorter linear cloning vector, coupled with a *ccdB* toxin gene, reduces the rate of negative clones and minimizes vector self-circularization. Finally, the recombinase system in Red/ET recombineering is strictly regulated by an arabinose-inducible promoter, which reduces unintended recombination events within the cloned gene cluster. To demonstrate the feasibility of their adapted approach, the researchers successfully cloned two gene clusters: the edeine biosynthetic pathway (49 kb) from *Brevibacillus brevis* X2340 and the bacillomycin biosynthetic pathway (37 kb) from *B. amyloliquefaciens* FZB42.[20] Both gene clusters were directly cloned and integrated into the chromosome of *B. subtilis* within a week. Remarkably, the researchers successfully achieved heterologous production of bacillomycin in the host strain. However, they encountered difficulties in heterologous expression of the edeine BGC in *B. subtilis*, presumably due to a mutation in the cloned gene cluster, the lack of certain essential precursors, or potential incompatibility between the promoter of the edeine BGC and *B. subtilis*.[20]

In 2015, Jiang *et al.* introduced a method termed CATCH (Cas9-assisted targeting of chromosome segments) for targeted cloning of bacterial genomic regions.[268] It involves cleaving specific DNA regions from intact bacterial chromosomes embedded in agarose plugs and ligating them with capture plasmids using Gibson assembly. CATCH demonstrates good efficiency in cloning genomic regions up to 100 kb in *E. coli*. The advantages of CATCH include reduced background DNA fragments due to in-gel Cas9 cleavage, which protects chromosomal DNA from mechanical shearing, and time-efficient *in vitro* circularization through Gibson assembly. Even though CATCH is less efficient for DNA segments longer than 100 kb, it is still suitable for cloning the majority of natural product BGCs since most are smaller than 100 kb.[242,269] This method has been used to successfully clone the 78 kb bacillaene BGC from *B. subtilis*. However, it is not yet compatible with heterologous expression. Over the past few years, several derivatives of the technique have been developed, some of which also directly enable heterologous expression of the captured BGCs, such as iCATCH[270] and CAT-FISHING.[271]

CAPTURE, or Cas12a-assisted precise targeted cloning using *in vivo* Cre-*lox* recombination, is another direct cloning technique that utilizes Cas12a instead of Cas9.[272] The process involves digesting genomic DNA with Cas12a-sgRNA, amplifying two capture plasmid fragments, each carrying a *loxP* site, assembling the digested genomic DNA and plasmid fragments using T4 polymerase exo + fill-in DNA assembly *in vitro*, and then introducing the pre-assembled linear products into an engineered *E. coli* strain for *in vivo* DNA circularization *via* Cre-*lox* recombination. Enghiad *et al.* successfully used this technique to clone 43 uncharacterized BGCs from various natural product classes, with sizes ranging from 10–113 kb.[272] These BGCs were subsequently heterologously expressed in either *S. avermitilis*, *S. lividans*, or *B. subtilis*, depending on the original host of the BGC, which was either a *Streptomyces* or *Bacillus* species. However, it is noteworthy that the heterologous expression of the five BGCs originating from *Bacillus* species did not yield the expected new compounds. The authors suggested that simply cloning the whole BGC was not sufficient to activate the silent gene clusters and that additional measures, such as introducing strong promoters or optimizing precursor availability would be needed for successful heterologous production. For the BGCs captured from *Streptomyces*, seven were detected by HPLC, and five of them were produced in sufficient quantities for structural characterization.[272]

A recently developed recombineering system for *B. subtilis*, based on the recombinase pair YqaJ/YqaK from a *B. subtilis* 168 prophage, can be used to insert large DNA sequences as well as introduce gene deletions in BGCs.[273] To achieve this, the transformation efficiency of *B. subtilis* was enhanced through overexpression of the ComK regulator. The system relies on the co-expression of *yqaJK* and *comK*, and utilizes a double-stranded DNA substrate with 100 base pair homology arms and a phosphorothioate modification at the 5′-end of the lagging targeting strand to improve recombination efficiency. This method offers a simpler and faster alternative to the labour-intensive preparation of single-stranded DNA substrates that are used in other recombineering methods.[274] Taken together, the YqaJK system proved to be superior to other recombinase systems previously

used in *B. subtilis*, making it an efficient genome manipulation tool for this species.[273]

4.3.3 Engineering tools. Various techniques exist to generate markerless mutations in *B. subtilis*.[275] One approach employs plasmid pDR244 from the *Bacillus* gene knockout library, which contains a temperature-sensitive ori and expresses the Cre recombinase gene.[276] Cre recognizes specific sequences, termed lox sites, which flank an integrated antibiotic cassette, enabling the removal of the cassette and leaving behind a lox scar.[276] Another method relies on the pMiniMAD plasmid, which carries a desired genetic alteration. This plasmid integrates into the host genome and is then excised using its temperature-sensitive ori, resulting in a markerless change.[42,277] The third and most recent method involves the use of CRISPR/Cas9 for genetic engineering in *B. subtilis*.

CRISPR-Cas technology has revolutionized the field of genome engineering by offering high editing efficiency and precision, cost-effectiveness, and ease of manipulation. While many CRISPR tools were initially developed for *B. subtilis*, their applications are expanding to other *Bacillus* species as well. Here, we will only briefly highlight the different systems that have been developed so far and provide some examples of their application in natural product BGC engineering. For more detailed reviews about CRISPR in *Bacillus*, we recommend the following reviews.[278,279]

Since its initial adaptation in 2016 as a genome-editing tool, CRISPR-Cas has undergone specific developments for metabolic engineering in *B. subtilis*.[39,280] This includes tools for generating both double-strand and single-strand breaks, which has enabled the introduction of point mutations, gene insertions, and gene deletions up to 38 kb. It has been successfully used in CRISPR interference (CRISPRi)[39] and CRISPR activation (CRISPRa)[281] systems for regulation of gene expression. Notable innovations include the Cas9 nickase (Cas9n)-variant for genome editing with reduced lethality[282,283] and the use of CRISPR effector proteins Cpf1 and MAD7 for genome and metabolic engineering purposes.[281,284] Additionally, the dCas9-AID tool enables single-base editing without the need for homologous recombination.[285] Multiplex CRISPR editing has been achieved with success, targeting up to six loci simultaneously for point mutations.[286] However, the current toolbox still lacks efficient multiplex CRISPR tools for other functionalities, such as knock-ins.[281]

Lui *et al.* recently showcased the application of CRISPR-Cas9 in cloning and enhancing the expression of a natural product BGC.[287] Although *B. subtilis* does not naturally produce lycopene, it possesses a native MEP pathway. This pathway assembles IPP and its isomer DMAPP, which are essential precursors for the biosynthesis of various compounds, including carotenoids like lycopene. Lui *et al.* first developed a versatile CRISPR/Cas9-based cloning toolkit for *B. subtilis*, comprising an optimized artificial exogenous gene insertion box, promoters, terminators, and guide RNA targets. The toolkit features six promoters of varying strengths, ranging from 0.9 to 23 times the potency of the commonly used promoter P_{43}. Additionally, seven highly efficient terminators were identified. A total of 13 key genes involved in the lycopene biosynthetic pathway were integrated into the *B. subtilis* genome at six specific sites and heterologously expressed to successfully produce lycopene. The researchers were even able to improve lycopene production further, achieving a 278.2-fold enhancement, amounting to 1.12 mg l^{-1}.[287]

In another example, the production of plipastatin in *B. subtilis* 1A751 was enhanced by disrupting the surfactin operon using CRISPR/Cas9. Plipastatin is synthesized by a NRPS and is considered to be a promising candidate for a range of applications, including biopesticides, fruit and vegetable preservation, cosmetics, and pharmaceuticals. Plipastatin and surfactin, which are both produced by *Bacillus* strains, share common biosynthetic precursors, such as glutamate, valine, and fatty acids.[288] By targeting and disrupting the *srfAB* and *srfAC* surfactin biosynthetic genes, more of these precursors were diverted to the plipastatin biosynthetic pathway, enhancing its yield.[289] While the gene editing efficiency was higher in the model strain *B. subtilis* 168, it was comparatively lower in the engineered strain 1A751-pps-srf, possibly due to its instability as a result of its extensive genomic modifications. Two novel plipastatins, along with nine known variants or derivatives, were identified and characterized. Moreover, a yield of 1600 mg l^{-1} plipastatin was reached, which is the highest reported yield to date.[289]

Engineering of NRPSs has been a longstanding goal in the field of natural products. Historically, strategies to engineer NRPSs, such as A domain modification and module exchanges, have often resulted in impaired or non-functional pathways. This recurring issue, often manifesting as undesirable assemblies, has been partly attributed to the extensive sequence repeats that are often present in NRPSs, leading to unwanted homologous recombination events. To overcome these challenges, structure-based approaches were developed, such as the Seamed Express Assembly Method (SEAM)" coupled with Ordered Gene Assembly in *B. subtilis* (OGAB) method.[290] The SEAM-OGAB method was applied to the plipastatin NRPS gene cluster from *B. subtilis*, which contains some of the most extensive direct-repeat sequences observed in any NRPS gene cluster, with 97% identity between repeating enzyme units. These repeated sections appear as module sequences throughout the A-T-C catalytic domains. In order to assemble the plipastatin BGC using SEAM-OGAB, each module was defined as an A-T-C/A-T-E-C unit. Extremely small (3 bp) seam overhangs were introduced into the C-A linkers based on homology. The seams were generated by introducing *Sfi*I restriction enzyme recognition sites, which then enabled gene assembly *via* the first-generation OGAB method. Since the plipastatin BGC consists of 10 C-A linker sequences, a total of 10 seams were required to reassemble the complete nucleotide sequence. Upon construction, the SEAM-OGAB-assembled plipastatin cluster, featuring multiple seams, was compared to the plipastatin BGC assembled without seams. Importantly, the introduction of seams was found to not impair the function of the NRPS. In a next step, modules were swapped between the plipastatin and surfactin NRPS, generating chimeric assembly lines capable of synthesizing hybrid lipopeptides. Overall, this approach enabled efficient and precise assembly of the gene cluster, paving the way for further studies on the functional

characterization and modification of the plipastatin NRPS, as well as other NRPS gene clusters with repetitive sequences.[290]

Bozhüyük *et al.* developed the concept of eXchange units (XUs)[291] and eXchange unit condensation domain (XUC)[292] to achieve efficient NRPS engineering. XUs leverage the modular structure of NRPSs but redefine modules as A-T-C units with a fusion site in the C-A linker region. The fusion site, typically an α-helix, can be identified *via* sequence alignment. Successful NRPS engineering through XU shuffling follows three key rules. First, XUs should ideally be mixed and matched within the same genera, as attempts to recombine NRPSs from distinct genera often lead to non-functional or impaired assembly lines. Second, the type of C domain should be considered, as changing the stereochemistry of a substrate significantly affects the productivity of engineered NRPSs. Finally, the acceptor-site specificity of the upstream C domain must be respected. While recent studies have questioned its role, adhering to this rule can guide engineering attempts. These rules improve the efficiency of engineering, although they become less relevant as the number of XUs decreases. However, it is important to note that while these rules enhance success rates, they are not absolute requirements for generating functional, engineered NRPSs.[293]

Another study has demonstrated the power of CRISPR-Cas9 gene editing for engineering complex NRPS assembly lines. Thong *et al.* successfully engineered NRPSs that were highly selective for specific amino acid substrates, producing new lipopeptides at levels comparable to those of the wild-type strain.[294] They found that different FSDs (Fused Salinomycin Domains) with identical selectivity but from different sources had varying effects on production titers, suggesting that subtle sequence and structural differences may influence compatibility. CRISPR-Cas9-mediated replacements proved to be faster, more efficient, and more accurate compared to conventional methods, making it feasible to screen and optimize multiple FSD exchanges.[294] So far, these methods have not been used in *Bacillus* species or used on NRPSs from *Bacillus*.

5 Conclusions and future perspectives

Over the past few decades, the identification of uncharacterized BGCs from various sources has become more easy due to the advances in sequencing technology and genome mining tools, such as antiSMASH.[295,296] However, accessing these BGCs presents many challenges, and heterologous expression has emerged as a successful strategy to circumvent some of these challenges. Until now, efforts have primarily focused on a subset of heterologous hosts, such as *Streptomyces* spp. and *E. coli*. However, there is significant potential in diversifying the pool of available host species and strains. This diversity can be valuable, especially when dealing with unknown mechanisms that impact heterologous expression. *B. subtilis*, with its inherent characteristics, is a promising host for heterologous expression of BGCs. In this final part, we aim to provide some guidelines for heterologous expression in *Bacillus*. First, we discuss the selection of an appropriate heterologous host. Secondly, we assess the suitability of different classes of BGCs for heterologous expression in *B. subtilis*. Lastly, we summarize the currently available and optimized *B. subtilis* host strains.

5.1 Where to find suitable BGCs for heterologous expression in *B. subtilis*

The selection of an appropriate host can significantly influence the success of heterologous expression. However, this choice is not always a straightforward decision. A key factor in maximizing the likelihood of successful heterologous expression is the close phylogenetic relationship between the host and the original producer. *Bacillus* species are prolific producers of bioactive natural products and show a high level of biosynthetic diversity, even among closely related strains.[297] A comprehensive analysis of *Bacillus* genomes, spanning 139 species, revealed an average presence of 11.6 putative BGCs per genome.[298] Marine-derived *Bacillus* and *Paenibacillus* strains in particular represent a rich source of novel chemistry.[299–301] Although interesting new secondary metabolites are discovered frequently,[302–305] research suggests that many *Bacillales* natural products remain undiscovered.[306,307] Furthermore, it has been shown that *Firmicutes* species, including many *Bacillus* strains, are often underrepresented in metagenomics libraries due to their ability to form endospores, which hinders the efficacy of standard DNA extraction techniques.[308] As a result, there is a vast untapped potential for discovering new chemistry within these taxonomic classes.

On the other hand, the taxonomic distance hypothesis, which suggests that the phylogenetic distance between the host and the producer predicts heterologous expression success, has been challenged.[261] While further research is necessary to understand the underlying mechanisms, it opens up a wider range of BGCs that can potentially be heterologously expressed. Low-GC BGCs from unrelated species may thus be feasible candidates for heterologous expression in *Bacillus*. In summary, the potential for heterologous expression in *Bacillus* remains largely untapped, although many research questions remain to be answered. Therefore, in the next two sections, we propose a framework of guidelines and conclusions addressing two important questions: which cryptic BGCs would be suitable for heterologous expression in *Bacillus*? Which *Bacillus* strain is the best host for a given BGC?

5.2 Assessing suitability of cryptic BGCs for heterologous expression in *Bacillus*

Currently, there is no universal one-size-fits-all host species for heterologous expression of all types of BGCs. In order to assess the suitability of a BGC for expression in *B. subtilis*, we summarize the current research findings for each class of natural products and identify areas where further pioneering studies are needed.

Firstly, we see many clear advantages for heterologous expression of terpenoid BGCs in *B. subtilis* due to its inherent ability to produce large amounts of isoprene *via* the MEP pathway. This metabolic pathway is responsible for producing isopentenyl diphosphate and dimethylallyl diphosphate, essential precursors for the biosynthesis of terpenoids (Fig. 3).[144]

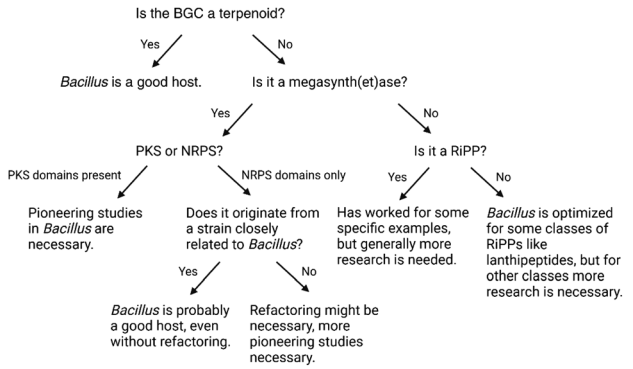


Fig. 3 Decision tree to verify whether *Bacillus* could be suitable for the expression of a given BGC.

For other natural product classes, selecting the right host strain is less clear-cut, although the inherent traits of *Bacillus* species provide a promising foundation for heterologous expression, regardless of the type of BGC. These include the ease of lab handling, natural competence, high transformation efficiency, thorough characterization of the metabolism, including native natural product biosynthesis, efficient secretion systems, and a strong track record in industrial production applications. Despite these advantages, *B. subtilis* has been underexplored as a host for heterologous BGC expression. Especially for PKS pathways, further research is required to uncover both the opportunities and challenges. On the other hand, heterologous expression of NRPS BGCs has been attempted and successfully achieved in several reported cases. Specifically, BGCs originating from taxonomically closely related species have shown high success rates and yields, often without the need for promoter replacement or other refactoring strategies. Additionally, BGCs from more distantly related gut species and even a fungus have been successfully expressed in *Bacillus*, albeit with some optimization. Overall, there is significant potential in using *B. subtilis* as a host for heterologous natural product BGC expression, though further research is needed to fully understand and address its limitations. One particular aspect is the size of the BGCs, as currently there has been no successful expression in *Bacillus* for BGCs larger than 50 kb.

With respect to RiPPs, *B. subtilis* has proven to be a very efficient host, particularly for lanthipeptide BGCs. Since the vast majority of these BGCs originate from Gram-positive species, using a *B. subtilis* host is a logical choice. However, one element that needs to be taken into account to ensure high yields is that the heterologous host must be resistant to the lanthipeptide being produced. Also other RiPP classes, naturally produced by both Gram-positive and Gram-negative bacteria, as well as compounds from marine prokaryotes have been successfully expressed in *Bacillus*. For BGCs that do not belong to any of these major classes, the feasibility of using *Bacillus* as a host must be evaluated on a case-by-case basis. Examples of some lesser-known natural product BGCs that have been successfully expressed in *Bacillus* species, include the rhizocticins and bacteriocins (pediocin).

5.3 Selecting the optimal *Bacillus* host for BGC expression

Various *B. subtilis* strains have been used for heterologous expression, with optimization efforts yielding significant improvements in lab handling. These improvements include increased transformation efficiencies[18,191] and disabled sporulation,[193,194] biofilm formation,[197,198] and autolysis[19,69,192] pathways to reach higher cell densities. In addition, several genes involved in the regulation of secondary metabolite production, such as *abrB*, *degQ* and *sfp* have been identified and studied.[20,50,134,196,204] Metabolic engineering strategies for yield optimization have been explored, albeit limitedly for some specific cases. This is particularly true for terpenoid biosynthesis, where efforts have been focused on overproduction of the starter enzymes of the MEP pathway, along with specific enzymes that are needed for the synthesis of specific terpenoids.[14,144] In addition, steps towards developing *Bacillus* strains with clean genetic backgrounds and reduced genomes have been taken.[54,75] However, demonstrated applications of these strains are still limited and their full potential is yet to be realized.

Different *Bacillus* strains have been optimized for different goals. Thus, we would like to offer guidance on selecting the optimal *Bacillus* strains for diverse purposes. For RiPP BGCs, the use of protease-deficient host strains is highly recommended for avoiding proteolytic degradation of the peptide product. Host strain options here include *B. subtilis* 1A751,[199] which is deficient in two proteases, *B. subtilis* WB800,[309] deficient in 8 proteases, and mini*Bacillus* PG10,[212] a strain with the added benefit of having a clean genetic background due to substantial genome reduction efforts.

Currently, there are no publicly available *Bacillus* strains that are specifically designed for terpenoid production. Most researchers start with a well-characterized *Bacillus* strain, such as *B. subtilis* 168, and then engineer it to suit their specific needs. This includes overexpression of certain genes or whole pathways, inactivating certain genes, generating mutant enzymes to increase fluxes, or fine-tuning the expression of genes or pathways by using specific promoters.

Up to now, the majority of megasynth(et)ase BGCs have been heterologously expressed in *B. subtilis* 168 or a derivative thereof, such as *B. subtilis* 1A751. The high transformation efficiencies of these strains facilitate the integration of these very large clusters. In addition, it is essential to repair the frameshift mutation in the *sfp* gene or integrate an *sfp* homologue to enable the production of NRPs, PKs, and hybrid metabolites. Although additional optimization strategies have been explored, this continues to be a topic for future research. The *B. subtilis* strain IIG-*Bs*20-4,[40] in which all native secondary metabolite BGCs have been removed, could also be a suitable host for heterologous expression. This strain is particularly interesting for uncharacterized BGCs, as it greatly facilitates compound detection and activity testing. However, the strain has so far only been used to assess surfactin production.[75]

While *Bacillus* has long been used for industrial enzyme production, there is room for optimization in the large-scale heterologous production of secondary metabolites. Known

high-yield strains have not yet been widely applied for heterologous BGC expression, and the currently-used strains may require additional optimization for this purpose. The body of research on how to increase surfactin yields in *Bacillus* can serve as a good starting point here. An example of an interesting strain in this context is the undomesticated *B. subtilis* ATCC 6051. This strain offers several advantages, such as high genomic stability during production due to its highly compromised natural competence levels, non-auxotrophy, the ability to reach high cell densities, and improved growth characteristics in complex media compared to *B. subtilis* 168.[192]

Furthermore, *Bacillus* strains that can be used for screening purposes, such as those designed for bacterial two-hybrid systems to study protein–protein interactions, have not yet been developed. Generating strains for these specific purposes in the future will further enhance the utility of *Bacillus* as a heterologous host for various applications.

In summary, *B. subtilis* has been shown to be a versatile host for natural product discovery through the heterologous expression of BGCs from various (meta)genomic sources. While specific classes of natural products, such as terpenoids, are particularly suited for expression in *Bacillus*, other classes require closer attention when selecting an appropriate expression host. In general though, it is clear that advances in host strain engineering and BGC cloning and refactoring have vastly improved the potential of using *B. subtilis* as a host for heterologous BGC expression.

6 Author contributions

H. P., H. G., H. V. C., M. F., Ja. M. and Jo. M. conceptualised the manuscript. H. P., H. G., and H. V. C. wrote the original draft. M. F., Ja. M. and Jo. M. reviewed and edited the manuscript.

7 Conflicts of interest

There are no conflicts to declare.

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