Microbial production of small peptide: pathway engineering and synthetic biology

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Summary
Small peptides are a group of natural products with low molecular weights and complex structures. The diverse structures of small peptides endow them with broad bioactivities and suggest their potential therapeutic use in the medical field. The remaining challenge is methods to address the main limitations, namely (i) the low amount of available small peptides from natural sources, and (ii) complex processes required for traditional chemical synthesis. Therefore, harnessing microbial cells as workhorse appears to be a promising approach to synthesize these bioactive peptides. As an emerging engineering technology, synthetic biology aims to create standard, well-characterized and controllable synthetic systems for the biosynthesis of natural products. In this review, we describe the recent developments in the microbial production of small peptides. More importantly, synthetic biology approaches are considered for the production of small peptides, with an emphasis on chassis cells, the evolution of biosynthetic pathways, strain improvements and fermentation.

Introduction
Natural products from plants or microorganisms are fertile sources of therapeutics and lead drugs, which have played a vital role in medicinal applications (Atanasov et al., 2015). Among these applications, antimicrobial agents derived from natural products have developed rapidly since penicillin was first discovered (Singh and Barrett, 2006). The remaining challenge is that new antimicrobial compounds are still urgently needed because drug resistance inevitably occurs with the extensive use of antibiotics. Small peptides are a group of natural products with relatively low molecular weights (< 10 kDa). These bioactive compounds can be classified into two main categories: ribosomally synthesized and post-translationally modified peptides (RiPPs) and non-ribosomal peptides (NRPs; Dang and Süßmuth, 2017; Fig. 1). The biosynthesis of RiPPs requires two steps: ribosomal synthesis of the precursor peptide and posttranslational modifications (PTMs; Montalbán-López et al., 2020). Due to differences in structural features (e.g., lanthionine), RiPPs are divided into different subfamilies, such as lanthipeptides. The structural diversity endows RiPPs with a broad range of bioactivities, including antifungal, antibacterial and antiviral activities (Amison et al., 2013). NRPs are a diverse family of natural products that are synthesized by large enzyme complexes known as non-ribosomal peptide synthetases (NRPSs; Podevels et al., 2008). NRPs are structurally diverse secondary metabolites with a variety of biological activities that can be exploited as therapeutic agents, such as the antibiotic daptomycin and the immunosuppressant cyclosporine A (Winn et al., 2016).

In recent decades, bioactive peptides have attracted increasing attention because they may bridge the gap between small molecules and protein drugs. Small
peptides have promising prospects in the pharmaceutical industry due to their broad-spectrum bioactivities, low toxicity and low propensity to induce the development of drug resistance, although further investigations are still needed to unravel their reliability (Barbosa et al., 2015). However, (i) the amount of small peptides available from natural sources is extremely low, and the inefficient isolation and extraction methods increase the difficulty of accessing these peptides. For example, the yield of the lanthipeptide lichenicidin produced by Bacillus licheniformis have just reached 4–6 mg l\(^{-1}\) (Kuthning et al., 2015), which has a huge gap compared with that of extracellular proteins, such as \(\alpha\)-amylase (17.6 g l\(^{-1}\); Niu et al., 2009). Meanwhile, (ii) bioactive peptides with sophisticated structures are particularly difficult to be produced using traditional chemical synthesis, which usually requires multiple reactions to introduce specific functional groups. As exemplified by lactocin S, a 34-residue lanthipeptide was isolated from Lactobacillus sakei L45, its biosynthesis involved only two enzymatic steps (modification, cleavage), after the prepeptide was synthesized in ribosome (Skaugen et al., 1997). The total synthesis...
of lactocin S required 71 steps using solid-supported synthesis (Ross et al., 2010). The intricate construction of cells may not only aid in normal cell metabolism but also serve as an arsenal to synthesize various secondary metabolites. In this regard, microbial production of bioactive peptides in diverse biosystems has been pursued as a potential approach. Although biosynthetic genes are ubiquitous in the currently sequenced genomes and transcriptomes of different microbes, the common feature of the complex biosynthetic mechanism remains unclear. In addition, compared to the other types of molecules, small peptide as a bioactive compound often triggers host immune responses, and its synthesis is thereby subjected to a more complicated regulation (Bartholomae et al., 2017; Yang et al., 2020). The manipulation of biosynthetic pathways encoded by large gene clusters is still a challenge due to the shortage of robust genetic tools and engineering strategy. Therefore, the development of engineered production platforms for the production of small peptides has been substantially hindered in the past few decades.

As a new discipline that combines biology and engineering, synthetic biology endeavours to create a standard, well-characterized and controllable synthetic system for the production of natural products. The developed chassis cells function as green manufacturing factories for the production of natural products of interest. Furthermore, the biosynthetic pathway encoding target products can be refactored by assembling a series of biological components based on the ‘bottom-up’ principle, and it can be precisely regulated in microbial cells in a reliable, predictable and standardized manner using robust genetic manipulation toolkits (Slusarczyk et al., 2012). Recently, synthetic biology has been valuable in the development of microbial platforms for the production of various natural products, including terpenoids (Zhang and Hong, 2020), flavonoids (Pandey et al., 2016) and polyketides (Cummings et al., 2014). In this review, attention has been paid to small peptides (3–50 amino acids) from microbial sources that act as antimicrobial substances and can be envisaged as potential therapeutic drugs. We describe the recent developments in the microbial production of small peptides using synthetic biology approaches.

**Chassis cells as hosts**

**Native hosts**

Microbes are capable of producing various necessary compounds to maintain growth and numerous unnecessary metabolites that are involved in defending against damage derived from extreme environments, such as limited nutrients. Small peptides are usually isolated from bacteria (e.g. Actinomycetes) or fungi (e.g. Aspergillus niger), and most of these peptides display antimicrobial activity that may contribute to viability by inhibiting the growth of other microorganisms (Dang and Süssmuth, 2017). Native hosts are desirable sources of drug-like small peptides because of their unique advantages, including the synthetic machinery, precursor supply, transport, self-resistance, modification and regulatory mechanisms, although a few biosynthetic gene clusters (BGCs) are silent under normal laboratory culture conditions. In addition, the expression of biosynthetic genes in heterologous hosts may fail because of the adaptive mechanism known as the restriction–modification (RM) systems of hosts (Mruk and Kobayashi, 2014). With these advantages, native hosts do not require additional modifications (i.e. the introduction of heterologous biosynthetic pathways) for the de novo synthesis of small peptides, suggesting that the challenge lies in improving peptide productivity. However, several limitations of native hosts should not be overlooked. For instance, most native hosts may not adapt to normal cultivation conditions, leading to slow growth, or may not be amenable to gene manipulations, which hampers the improvement in host production performance through bioengineering strategies.

**Heterologous hosts**

Regardless of whether metabolic engineering strategies or synthetic biology approaches are used to produce compounds of interest, some ideal host traits are required to balance growth and genetic modification. Therefore, a promising strategy is to develop surrogate hosts for peptide biosynthesis. In general, these heterologous hosts would possess the following collective characteristics: (i) a clear genetic background, (ii) tractable gene manipulation, and (iii) ease of cultivation. Moreover, some traits that are similar to the native hosts might facilitate heterologous expression. For example, *Streptomyces* are the main sources of secondary metabolites that are synthesized by large-sized gene clusters, and strains might provide various precursor pools and extensive modification machineries. These ideal traits make them potential chassis cells for the production of natural peptide products through the catalysis of a series of enzymes encoded by multiple genes. A representative example is *Streptomyces coelicolor*, a well-known host that is widely used to produce natural products (Gomez-Escribano and Bibb, 2011). In addition, pathway-specific biology must be considered because some enzymatic reactions required for peptide biosynthesis rely on specific cellular structures (Hoppert et al., 2001; Meijer et al., 2010). As shown in the study by Gidijala et al. (2009), penicillin production is lower in a yeast strain lacking peroxisomes. The rapid progress in
genetic manipulation technologies, including the CRISPR/Cas 9 system, along with the increased availability of synthetic biology tools has promoted the cloning and refactoring of BGCs of natural products in heterologous hosts. The biosynthesis of small peptides has been successfully achieved by harnessing various model hosts, such as *Escherichia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae* (Table 1). These well-characterized hosts are beneficial not only for elucidating the catalytic mechanisms of biosynthetic enzymes via protein expression engineering but also for improving productivity based on genetic engineering. Furthermore, on this basis, synthetic chassis cells could be designed through the optimization of these heterologous hosts by using standard elements and rational engineering strategies (Adams, 2016). These synthetic chassis cells could serve as potential microbial cell platforms for large-scale production of various desired products. Here, chassis cells with specific traits for the biosynthesis of small peptides are proposed (Table 2).

**Artificial evolution of biosynthetic pathways**

*Pathway design*

**Identification of biosynthetic pathways.** Small peptides are synthesized by specific biosynthetic pathways composed of various enzymes encoded by the corresponding gene clusters within the chromosome of native hosts. The identification of biosynthetic pathways is crucial to improve our understanding of synthetic machinery, for the discovery of new compounds, and for heterologous expression (Amagai et al., 2017; Kawahara et al., 2018; Shi et al., 2019). The plummeting cost of genome sequencing has facilitated the increase in the number of sequenced bacterial genomes, which provide a wealth of data for investigating potential BGCs encoding biosynthetic pathways (Vallenet et al., 2009). Due to advances in bioinformatics techniques coupled with the interest in peptide biosynthetic logics, many bioinformatics tools have been developed that enable researchers to mine BGCs of peptide natural products by matching the biosynthetic genes to the most similar candidates through a search of known databases, such as the antiSMASH database (Blin, Medema, et al., 2017). These computational tools are dedicated to the identification and characterization of the BGCs of peptides and other natural products in different species (Fig. 2a), including bacteria (Blin, Wolf, et al., 2017), fungi (Khalid et al., 2010) and plants (Kautsar et al., 2017). Some of the tools based on specific algorithms have the ability to predict chemical structures based on detected biosynthetic genes. For instance, PRISM 3 is an available web server that predicts the chemical structures of natural peptide products based on homology to known BGCs or biosynthetic enzymes by modelling the structure scaffold as a chemical graph (Skinnider et al., 2017). Recently, mass spectrometry (MS)-based genome mining has been employed to identify the BGCs of secondary metabolites. Pep2Path, an automated mining tool based on advanced MS detection equipment, precisely links natural peptide products with the likely BGCs according to their conserved biosynthetic logics (Medema et al., 2014). Pep2Path consists of RiPP2Path and NRP2Path, both of which are able to convert a detected mass shift into a possible amino acid sequence tag that can be further used to match the candidate gene clusters. Unlike RiPPquest, an algorithm that is dedicated to identifying the prepeptide-encoding genes of RiPPs (Mohiman et al., 2014), RiPP2Path is suitable for all RiPPs. Additional genome mining techniques for natural peptide products have previously been reviewed in detail elsewhere (Boddy, 2014; Hetrick and van der Donk, 2017). All of these user-friendly tools not only accelerate the discovery of BGCs for small peptides and other secondary metabolites but also provide avenues for the production of natural products by expressing BGCs in heterologous hosts.

**Refactoring of biosynthetic pathways.** The gene clusters responsible for the biosynthesis of small peptides are composed of multiple operons. The native BGCs may be subjected to endogenous cellular regulation, which will affect the levels of gene expression within the biosynthetic pathway (Shao et al., 2013). A controllable biological process that is based on the rewiring of BGCs with well-characterized DNA parts is required to circumvent this limitation (Larroude et al., 2018). This process might be facilitated by refactoring, in which native biosynthetic pathways are reassembled using genetic parts (Smanski et al., 2016; Fig. 2b). During the process of refactoring, non-coding genes and native regulatory elements within the BGC are removed. Furthermore, the uncharacterized regulatory elements within the coding DNA sequences (CDSs) will be identified and deleted by randomly assembling coding genes in silico. The remaining coding genes are obtained using DNA synthesis techniques. By refactoring the BGCs, the expression of genes is controlled using diverse biological parts, such as the promoter, RBS, terminator (Khalil and Collins, 2010). Expanded parts, such as insulators, are used to construct synthetic pathways because of their ability to increase the reliability of the functions of each part and to predict the behaviour of synthetic circuits in different genetic contexts (Lou et al., 2012). The BGCs that are equipped with synthetic regulatory elements are theoretically relatively orthogonal, which would allow natural
Table 1. Common strategies used for the biosynthesis of small peptides in heterologous hosts.

| Peptide                          | Characterization                    | Native host          | Heterologous host | Strategy                  | Yield                        | References                  |
|----------------------------------|-------------------------------------|----------------------|-------------------|---------------------------|-----------------------------|-----------------------------|
| Ruminococin A                    | RIPP, antibacterial agent (e.g. against Clostridia) | Ruminococcus gnavus  | E. coli           | Semi-in vitro biosynthesis | 6 mg l⁻¹ (prepeptide)       | Ongey et al. (2018)         |
| Bovicin HJ50                     | RIPP, antibacterial agent (e.g. against B. subtilis) | Streptococcus bovis  | E. coli           | Semi-in vitro biosynthesis | 300 µg l⁻¹                  | Xiao et al. (2004); Lin et al. (2011) |
| Pallidocin                       | RIPP, antibacterial agent (e.g. against Caldibacillus sp.) | Aeribacillus pallidus | E. coli           | Semi-in vitro biosynthesis | ~150 µg l⁻¹ (active peptide) | Kaunietis et al. (2019)     |
| Nisin A                          | RIPP, antibacterial agent (e.g. against Listeria Monocytogenes), commercialization | Lactococcus lactis  | B. subtilis       | Integration of the intact nisin gene cluster into the genome | —                           | Yuksel and Hansen (2007)    |
| Peptide          | Characterization                                                                 | Native host | Heterologous host | Strategy                                                                                           | Yield            | References                                                                 |
|------------------|----------------------------------------------------------------------------------|-------------|-------------------|---------------------------------------------------------------------------------------------------|------------------|---------------------------------------------------------------------------|
| Mersacidin       | RIPE, antibacterial agent (e.g. against Staphylococcus aureus)                    |             | B. amyloliquefaciens | • Expression of some biosynthetic genes (structural gene and the specific regulator)               | —                | Barbosa et al. (2015); Herzner et al. (2011)                              |
| Streptocollin    | RIPE, enzyme inhibitor (e.g. protein tyrosine phosphatase 1B)                    | S. coelicolor | S. coelicolor     | • Transferring the cosmid containing the intact BGC into heterologous hosts                       | 5.4 mg l⁻¹ (S. coelicolor M1152) | Iftime et al. (2015)                                                      |
| Blia             | RIPE, antibacterial agent (e.g. against L. monocytogenes)                        | B. licheniformis | E. coli | • Expression of biosynthetic genes encoding Blia and Blij based on the two-plasmid system        | 4 mg l⁻¹ (Blia)  | Begley et al. (2009); Kuthning et al. (2015)                              |
| Lichenicidin     |                                                                                  |             |                   |                                                                                                   | 6 mg l⁻¹ (Blĳ)   |                                                                           |
| Peptide       | Characterization                          | Native host                               | Heterologous host | Strategy                                                                 | Yield         | References                  |
|--------------|-------------------------------------------|-------------------------------------------|-------------------|---------------------------------------------------------------------------|---------------|-----------------------------|
| Chaxapeptin  | RiPP, inhibit cell invasion               | *Streptomyces leeuwenhoekii*              | *E. coli*         | Expression of the complete gene cluster using a plasmid vector           | 0.1 mg l⁻¹    | Martin-Gómez et al. (2018)  |
| Fuscanodin   | RiPP                                      | *Thermobifida fusca*                      | *E. coli*         | Expression of the entire gene cluster using the plasmid vector          | —             | Koos and Link (2019)        |
| GE2270A      | RiPP, antibacterial agent (e.g. against *S. aureus*) | *Planobispora rosea*                      | *S. coelicolor*   | Expression of the gene cluster that deleted the ribosomal genes flanking the gene cluster | 1.75 mg l⁻¹    | Flinspach et al. (2014)     |
| Peptide                  | Characterization                        | Native host     | Heterologous host | Strategy                                                                                       | Yield | References                        |
|-------------------------|-----------------------------------------|-----------------|-------------------|-----------------------------------------------------------------------------------------------|-------|-----------------------------------|
| GE37468                 | RiPP, antibacterial agent (e.g. against S. aureus) | Streptomyces    | S. lividans       | Expression of the intact gene cluster based on the E. coli-Streptomyces shuttle vector         | 2–3 mg l\(^{-1}\) | Young and Walsh (2011)            |
| Telomestatin            | RiPP, telomerase inhibitor              | Streptomyces anulatus | S. avermitilis   | Expression of the intact gene cluster based on the BAC vector                                 | 5 mg l\(^{-1}\) | Amagai et al. (2017)              |
| Penicillin G            | NRP, antibiotic, commercialization      | Penicillium chrysogenum | S. cerevisiae    | Integration of the NRPS gene and the NRPS activator gene into the genome and expression of the remaining genes using the plasmid vector | 3 ng l\(^{-1}\) | Awan et al. (2017)                |
| Peptide          | Characterization                      | Native host       | Heterologous host | Strategy                                                                                     | Yield          | References          |
|------------------|---------------------------------------|-------------------|-------------------|--------------------------------------------------------------------------------------------|----------------|---------------------|
| Indigoidine      | NRP, pigment                          | *Streptomyces lavendulae* | *S. cerevisiae*   | • Integration of the NRPS BpsA and the sfp gene encoding 4’-phosphopantetheinyl transferase (PPTase) into the genome | 980 mg l⁻¹     | Wehrs et al. (2018)  |
| Beauvericin      | NRP, antibiotic, anthelmintic, cytotoxic, et al. commercialization | *Beauveria bassiana* | *Aspergillus niger* | • Expression of the gene cluster encoding synthetases under control of the Tet-on inducible promoter | 628.4 ± 211.1 mg l⁻¹ | Boecker et al. (2018) |
| Echinomycin      | NRP, antitumor                         | *Streptomyces lasaliensis* | *E. coli*         | • Refactoring the BGC with the T7 promoter and T7 terminator  
• Expression of the gene cluster and the sfp gene encoding PPTase based on the multi-plasmid system  
• Using orthogonal origins of replication and antibiotic resistance genes for the stable retention of plasmids | 0.3 mg l⁻¹     | Watanabe et al. (2006) |
regulatory networks to be bypassed and force the expression of the controlled genes (Myronovskyi and Luhzhetskyy, 2016). Another advantage is the potential application of high-throughput techniques to the refactored BGCs for the optimization of biosynthetic pathways. For example, synthetic promoter libraries have been established to refactor the biosynthetic pathway for the production of desired products based on high-throughput screening (Siegl et al., 2013). Different promoters that vary in strength enable the optimization of BGC expression through a rational combinatorial strategy (Awan et al., 2017). MacPherson et al. constructed a series of short synthetic terminators by randomly linking terminators with a consensus sequence. These synthetic short terminators avoided repetitive sequences and improved the stability of recombinant synthetic circuits and the assembly of biosynthetic pathways into S. cerevisiae (MacPherson and Saka, 2017). Additionally, refactoring permits the creation of new structures by swapping genetic modules encoding diverse modification enzymes. This strategy is particularly important for natural products with similarly biosynthetic mechanisms. For instance, NRPSs are usually composed of multiple modules that are responsible for integrating diverse monomers into the peptide backbone, which facilitates the production of new chemical structures through the modification of biosynthetic modules (Nguyen et al., 2006). The state-of-the-art structures are generated through the combination of different biosynthetic modules derived from the BGCs of non-ribosomal peptides (Zobel et al., 2016). Furthermore, hybrid enzymes that are capable of synthesizing new compounds have been generated by the fusion of NRPS with other megaenzymes, such as polyketide synthases (PKSs; Zhang et al., 2020).

Pathway construction

In general, two strategies have been developed for the construction of biosynthetic pathways. The first is de novo DNA synthesis. The availability of sequenced genomes along with advanced gene mining tools has allowed biologists to access the BGCs of natural products. Furthermore, the cost of DNA synthesis has decreased, which has facilitated the construction of biosynthetic pathways through commercial synthesis. Researchers were able to perform de novo synthesis of codon-optimized large fragments that closely match the codon usage of heterologous hosts. The other strategy for pathway construction is DNA assembly (Fig. 2c). Many assembly methods are available and have been reviewed, including Gibson assembly, ligase cycling reaction, scarless stitching and Golden Gate assembly (Kosuri and Church, 2014). When the BGCs

Table 1. (Continued)

| Peptide | Heterologous host | Native host | Strategy | Yield          | References |
|---------|-------------------|-------------|----------|----------------|------------|
| Enniatin B | Filamentous fungi (e.g., Aspergillus) | B. subtilis | Expression of the NRPS gene using chromosome integration and plasmid-based inducible expression systems | 1.1 mg l\(^{-1}\) | Zobel et al. (2015); Boecker et al. (2018) |
| NRP, antibiotic, cytotoxic | | | Codon optimization | | |

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are obtained, robust vectors are necessary to carry them. Common tools include cosmids and fosmids, which have been used to clone and express the large gene clusters of small peptides (Caetano et al., 2011; Shi et al., 2019). The expression of the intact gene clusters based on multiplasmid systems is also an effective strategy for the manipulation of large gene clusters (Watanabe et al., 2006). In addition, bacterial artificial chromosome (BAC) vectors have been applied to express all BGCs of secondary metabolites (Liu et al., 2009). BACs containing the ϕC31 attB and oriT functions have been used to integrate large gene clusters into the chromosome of heterologous hosts (Baltz, 2012). This feature has been employed to construct small peptide-producing strains by inserting the BGCs into the chromosomes of the heterologous hosts (Alexander et al., 2010). Advances in next-generation sequence sequencing technologies known as ‘plug-and-play’ approaches are required to express orphan pathways from sequenced microorganisms for the biosynthesis of natural products of interest, which enable the construction of novel vectors that are capable of directly cloning, refactoring, and expressing large gene clusters for the design, construction, and discovery of novel secondary metabolites (Yamanaka et al., 2014). Recently, synthetic vectors have been developed due to their abilities to construct artificial biosynthetic pathways and control the expression of genes by integrating various biological parts (Pandey et al., 2016). As a versatile platform for manipulating biosynthetic pathways involved in multiple genes, the ePathBrick vector system supports the modular assembly of pathway genes using multiple compatible plasmids simultaneously and allows the fine-tuning of gene expression by controlling transcription signals (Xu et al., 2012).

For the biosynthesis of RiPPs synthesized via the ribosomal machinery, specific structures (e.g. lanthionine) are essential for the bioactivity of RiPPs, which are formed by modifying enzymes that are capable of recognizing conserved sequences of precursor peptides. The in vivo PTMs will not be obstructed when functional tags are inserted into the N-terminus of the leader peptide (Huo and Donk, 2016). Based on this mechanism, semi-in vitro biosynthesis (SIVB) might be an alternative strategy for producing RiPPs using the artificial prepeptide chain (Fig. 2d). The modified prepeptides are produced by expressing the precursor peptide in the form of a fusion protein with an N-terminal tag, followed by the activation of the modified prepeptide in vitro through the cleavage of the leader peptide by a specific endonuclease (Lin et al., 2011). The modified prepeptide may provide the desired properties following the introduction of various functional tags, such as His tags (Zambaldo et al., 2017).

Pathway optimization

The challenge for the production of natural products is developing a method to control the biosynthetic flux in synthetic biosystems. In synthetic biology, the regulation of biosynthetic pathway has been achieved at the transcriptional and translational levels. The expression of genes within the biosynthetic pathway is precisely controlled using various genetic parts (Seo et al., 2013). A common strategy for the biosynthesis of small peptides is to increase the expression of the genes related to biosynthesis by inserting strong promoters in front of the operons (Qiu et al., 2014; Iftime et al., 2015; Ji et al., 2015; Ni et al., 2017; Wang et al., 2018). The constitutive promoter ermE*, which is derived from the native promoter of the erythromycin-resistance gene, is widely used to drive gene or gene cluster expression for the production of small peptides (Thykaer et al., 2010; Flinspach et al., 2014; Iftime et al., 2015). The ability to control the levels of gene expression via inducible promoters is an alternative temporal strategy for coordinating cell growth and product formation (Li et al., 2018a). A representative example is the T7 promoter, which has been used to increase the expression of biosynthetic genes in E. coli for the heterologous production of peptides (Weiz et al., 2011; Kuthning et al., 2015). The translational regulation of the biosynthetic pathway has been achieved by modulating the efficiency of protein synthesis (Thiel et al., 2018). For instance, Martin-Gómez and colleagues substituted native RBS sequences with E. coli-optimized RBS sequences in front of the biosynthetic genes cptB1 and cptB2 within the BGC of the lasso peptide chaxapeptin, resulting in chaxapeptin production in E. coli (Martin-Gómez et al., 2018).

Although the overexpression of biosynthesis-related genes might efficiently increase productivity in most cases, a prerequisite for pathway optimization is the balanced expression of genes in operons (Pfleger et al., 2006). The challenge is that each gene might be subjected to complex regulations in the cell and the optimized expression levels of gene clusters are unknown, which might further hamper the increased production of the molecules of interest. This problem might be circumvented through the refactoring of BGCs based on the libraries of genetic parts (Ji et al., 2018). The use of diverse regulatory parts in a combinatorial manner may exert positive synergistic effects on the optimization of biosynthetic pathways (Kosuri et al., 2013). Meanwhile, genetically encoded biosensors coupled with high-throughput fluorescence-activated cell sorting (FACS) have provided a method to screen or select desired metabolites at the single-cell level.
require detailed knowledge of the biosynthesis in the be used to refactor the BGCs of interest and does not egy allows other genetic regulatory parts (e.g. RBSs) to screened in an appropriate host. Furthermore, this strat- would enable the ideal expression levels to be researchers to construct a library of bot clusters that attenuated with a combination of variations from libraries of synthetic parts will guide the design of synthetic pathways using computational methods (Rodrigo et al., 2011). As exemplified in the biosynthesis of penicillin in yeast, Awan and coworkers optimized the production of the non-ribosomal peptide penicillin by constructing and testing hundreds of different combinations of penicillin pathway genes with different promoters with varying strengths and expression dynamics (Awan et al., 2017). Recently, a random rational strategy was applied to engineer the gene cluster of bot-tromycin for the construction of overproducing strains. The cluster was refactored with a combination of various promoters with unknown strengths, which allowed researchers to construct a library of bot clusters that would enable the ideal expression levels to be screened in an appropriate host. Furthermore, this strategy allows other genetic regulatory parts (e.g. RBSs) to be used to refactor the BGCs of interest and does not require detailed knowledge of the biosynthesis in the initial stage (Horbal et al., 2018).

Strain improvement

Activation of positive regulators

The improvement of strain performance can be achieved within the bio-based peptide-producing system through the use of pathway engineering strategies, such as transcription regulation (Fig. 3). The biosynthesis of RiPPs is regulated by complex regulatory mechanisms that are commonly mediated by specific pathway regulators (Bartholomae et al., 2017). For example, the biosynthesis of the class I lanthipeptide nisin is regulated by quorum sensing, which is mediated by the two-component system nisRK, the specific regulatory genes within the gene cluster (Lubelski et al., 2008). Specific pathway regulators control the transcription of biosynthetic genes within the gene clusters by binding to the promoter regions of operons (Zhang et al., 2014). The overexpression of these positive regulators increases the transcription of structural genes within gene clusters and improves productivity (Li et al., 2018b). In addition to pathway-specific regulators, global regulators also exert positive effects on the biosynthesis of RiPPs. An example is microcins, a class of RiPPs with a low molecular weight that are produced by E. coli and related Enterobacteria (Severinov et al., 2007). The transcription of the microcin C51 operon is regulated by RNA polymerase sigma S factor (σ^S) and the global transcriptional regulator CRP that interacts with cyclic AMP in the cyclic complex CRP-cAMP. The production of microcin C51 is markedly decreased or abolished upon the mutation of σ^S, CRP or cAMP (Fomenko et al., 2001).

Table 2. The proposed chassis cells for the production of small peptides.

| Chassis cell characterization | Advantage | Engineering strategy | References |
|-----------------------------|-----------|----------------------|------------|
| Genome reduction | • Fewer unexpected metabolites | • Gene deletion | Gao et al. (2010) |
| | • Lower energy consumption | • Chemical synthesis of the specialized genome |
| | • Blocking of potential competing pathways | | |
| | • Increasing availability of precur-sors | | |
| | • Avoid degradation by endoge-nous proteases | | |
| Increasing resistance | • Tolerance for peptide toxicity | • Genome shuffling | Yu et al. (2018); Hu and Ochi (2001); Huo et al. (2012); Zhang et al. (2016); Huo et al. (2012); Qi et al. (2017); Dorr et al. (2016); Cao et al. (2018) |
| | • Tolerance to varied fermentation condition | • Screening of drug-resistance mutations |
| | | • Increased expression of immunity genes |
| | | • Engineering transporters |
| | | • Control of resistance genes transcription |
| Secretion remodelling | • Avoid excess accumulation of peptide in cell | • Cell wall modification | Kuipers et al. (2004); Li et al. (2015); Kuipers et al. (2006) |
| | • Improve productivity | • Engineering transporters |
| | | • Engineering secretory systems, for example Sec pathway | |

(Hossain et al., 2020). A practical application is that cells producing high levels of the desired metabolites are selected via a biosensor-based high-throughput screen without knowing the expression level of each gene within the biosynthetic pathway (Wang et al., 2019). The dynamic regulation of the biosynthetic pathway is necessary for the optimal production of secondary metabolites, while avoiding the excess accumulation of intermediates, especially toxic compounds (Dahl et al., 2013; Xu et al., 2014). The well-characterized dynamic behaviours of synthetic circuits from libraries of synthetic parts will guide the design of synthetic pathways using computational methods (Rodrigo et al., 2011).

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The activation of positive regulators of specific pathways is also essential to increase biosynthetic gene transcription and increase production in the biosynthesis of NRPs. The gene cluster responsible for the synthesis of the glycopeptide teicoplanin is regulated by two positive regulators, tei15* and tei16*, which are StrR- and LuxR-type transcriptional regulators, respectively. The expression of tei15* and tei16* under the aac(3)IV promoter in Actinoplanes teichomyceticus increases the production of teicoplanin (Horbal et al., 2014). Daptomycin is a cyclic lipopeptide produced by Streptomyces roseosporus that may represent a clinical therapeutic agent for the treatment of skin infections (Baltz, 2010). Yuan and coworkers confirmed that DepR1, a TetR family transcriptional regulator, is a positive regulator of daptomycin biosynthesis. DepR1 might mediate the positive feedback regulation of daptomycin production by directly binding to its own promoters. The overexpression of DepR1 results in the improved production of daptomycin and a shorter fermentation period (Yuan et al., 2016). Unlike the action of DepR1, the TetR family transcriptional regulator AtrA regulates the production of daptomycin by activating the A-factor signalling pathway (Mao et al., 2015). Additionally, specific molecules also serve as positive regulators to promote biosynthetic gene transcription by mediating signal transduction. An example is the biosynthesis of the non-ribosomal peptide surfactin, which is produced by B. subtilis through a process by a cell density-responsive mechanism. The specific extracellular signalling peptides comX and phrC induce the expression of quorum-responsive genes (srfA). The overexpression of comX and phrC increases the yield of surfactin (Jung et al., 2012).

Disruption of negative regulators

The negative regulators will downregulate the expression of biosynthetic genes by binding to the promoter of biosynthetic genes, leading to a decrease in the production of target products (Reverchon et al., 2002). The removal of these negative regulators could relieve the transcriptional repression of the BGC and contribute to peptide synthesis (Choi et al., 2009). For example, the ArsR family transcriptional regulator DepR2 represses the expression of the daptomycin gene cluster by interacting with the dptEp promoter. The deletion of depR2 caused an approximately 2.5-fold increase in the production of daptomycin (Mao et al., 2017). The biosynthesis of peptide antibiotics is related to the endogenous metabolite states of the cell, such as sporulation (Marahier et al., 1993). AbrB is a global regulator that controls the expression of genes involved in the transition state between the postexponential phase and stationary phase and sporulation in B. subtilis (Strauch et al., 1989). Moreover, AbrB plays an important role in the production of antibiotics and degradative enzymes and many other physiological processes in B. subtilis (Phillips and Strauch, 2002). AbrB-encoding genes have been identified in various bacterial species, including archaea (http://mbgd.genome.ad.jp/ Chumsakul et al., 2011). In a previous study, researchers introduced the BGC of polymyxin into B. subtilis and successfully observed product generation. However, quantitative real-time PCR results indicated that the transcription level of the lipopeptide antibiotic polymyxin biosynthetic gene pmxA was reduced in the presence of AbrB. Transcription was significantly recovered upon the deletion of the abrB gene encoding the AbrB regulator, resulting in the efficient production of polymyxin (Park et al., 2012). The unexpected repressive effects of the AbrB regulator on the expression of biosynthetic genes have also been observed in the biosynthesis of RiPPs in B. subtilis. For example, the biosynthesis of the lantibiotic subtilin was repressed by the transcriptional regulator AbrB, which caused a low level of expression of biosynthetic proteins during the exponential growth phase. The production of subtilin was substantially increased by deleting of the abrB gene in B. subtilis ATCC 6633 (Stein et al., 2002). Additionally, global regulators that are involved in primary metabolism may exert negative effects on the biosynthesis of secondary metabolites. For example, the two-component system PhoR-PhoP, which is related to phosphorus metabolism, was proven to suppress antibiotic synthesis in Streptomyces lividans and the deletion of phoP or phoR-phoP relieved this repression (Sola-Landa et al., 2003). Deletion of the phoP gene in B. licheniformis also increased the yield of bacitracin, a NRP produced by B. subtilis and B. licheniformis (Cai et al., 2019).

Precursor supply

For the biosynthesis of RiPPs, the building blocks are usually proteinogenic amino acids and unnatural amino acids that are incorporated into the peptide backbone through amber-suppression technology (Himes et al., 2016). In addition to proteinogenic/non-proteinogenic amino acids (Hubbard et al., 2000), some specific moieties may be incorporated into the non-ribosomal peptide backbones, such as fatty acids (Wu et al., 2019). Here, the emphasis is placed on the precursors supplied for the biosynthesis of NRPs (Fig. 3). The supply of precursors is increased through rational genetic engineering to perturb metabolic fluxes, including (i) the overexpression of enzymes involved in the precursor synthesis pathway (Thykaer et al., 2010), (ii) the disruption of competing pathways or by-product pathways (Lee et al., 2015), and (iii) the manipulation of transcriptional regulators related
to primary metabolism (Cai et al., 2019). Recently, a promising synthetic biology approach was reported to increase the precursor supply through the redesign of a biosynthetic pathway by replacing native enzymes with heterologous proteins from unrelated pathways. Hydroxyphenylglycine (HPG), the precursor for the calcium-dependent antibiotic of S. coelicolor, was synthesized efficiently using this method (Diez et al., 2015).

Redirecting metabolism

Engineering cell microbial factories for bioproduction requires to overcome metabolic burden which may place hidden constraints on host productivity (Wu et al., 2016). Progress in systems biology and the development of various omics techniques (e.g. metabolomics), researchers are able to analyse the metabolic levels of biosynthetic pathways from a global perspective (Wang et al., 2020). One example is the establishment of metabolic network models for in silico analyses of metabolic flux (Ma et al., 2018). To date, genome-scale metabolic network model (GMM) reconstruction has been completed in various strains, such as S. coelicolor (Borodina et al., 2005). The metabolic network model is able to assess the metabolic capabilities and investigate flux bottlenecks through computational simulations (Burn Kim et al., 2004). As exemplified in the production of daptomycin, the metabolic flux of each reconstituted strain was compared with the wild-type strain, and three potential targets (zwf2, dptI, and dptJ) related to the production of daptomycin were identified. The overexpression of these three target genes individually increased the production of daptomycin. Furthermore, compared with the parental strain, the strain coexpressing these three genes exhibited a 34.4% increase in daptomycin yield, suggesting that the enzymes encoded by these three genes exert a synergistic effect on daptomycin biosynthesis (Huang et al., 2012). Advances in gene editing tools and DNA synthesis enable multiplex genome engineering of industrial chassis organisms for optimize bioproduction (Barbieri et al., 2017; Fig. 3). Multiplexed automated genome engineering (MAGE) permits access to a library of mutants with diverse genotypes by introducing oligonucleotides, including insertions, deletions and mismatches.
The combination of this technique with suitable screening approaches could be utilized to investigate target strains that possess optimized in vivo metabolic fluxes for target products. Recently, the CRISPRi-based programmable circuit was designed to regulate the metabolic pathway, completing dynamic and self-regulatory dual control of metabolic flux without external inducers (Wu et al., 2020).

**Organelle engineering**

Subcellular organelles play vital roles in the biosynthesis of secondary metabolites based on the spatial distribution of enzymes in eukaryotes (Keller, 2015; Hammer and Avalos, 2017). As an example, peroxisomes are nearly ubiquitous single-membrane organelles involved in the biosynthesis of fungal metabolites (Stehlik et al., 2014). The biosynthesis of penicillin and related cephalosporins requires specific enzymatic reactions within the peroxisomes (Bartoszewska et al., 2011). This suggests that subcellular compartmentalization of enzymes involved in secondary metabolites biosynthesis may have a promising engineering potential (Fig. 3). Herr et al. tested the peroxisome targeting strategy for the localization of main enzymes of penicillin biosynthesis in *A. nidulans*. A peroxisomal targeting signal 1 (PTS1) tag was incorporated into the non-ribosomal peptide synthase AcvA, which led to a 3.2-fold increase in penicillin production. Moreover, increased peroxisomes through overexpression of *pexK* also led to improvement in penicillin production (Herr and Fischer, 2014). The biosynthesis of RiPPs is mainly involved in the ribosome translation with the incorporation of 20 canonical amino acids into the peptide chain. The non-canonical amino acids (ncAAs) as building blocks are available through the interference of translation process via ribosome, which paves the way to expand structural diversity of ribosomal peptides (Budisa, 2013). For example, Tianero et al. (2012) revealed that the specific ncAA can be incorporated into the precursor peptide of cyanobactin using an orthogonal tRNA/aaRS pair in response to the nonsense or frameshift mutation. Recently, it was demonstrated that ribosome engineering exerted tremendous effects on the production of secondary metabolites (Lopatniuk et al., 2019). Several results showed that ribosomal protein S12 was related to drug resistance of producer and mutations in the ribosomal protein S12 efficiently increased small peptide production (Huo et al., 2012; Wang et al., 2014).

**Fine-tuning fermentation**

Fermentation is an important part of bio-based production using microbial hosts as cell factories. The common strategies including medium optimization (Dang et al., 2019), fed-bath fermentation (Costas Malvido et al., 2016), and control of parameters such as dissolved oxygen (DO; Guez et al., 2008) have been employed to improve productivity of small peptide-producing strains. However, the maximum potential is difficult to exploit

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and achieve the highest productivity in the scaled fermentation process. One reason is that traditional methods for controlling fermentation mainly rely on empirical knowledge based on the metabolic states of the wild-type strain, which poses a challenge to accurately regulate cellular metabolic states for high productivity. The artificial chassis cells designed for specific purposes (e.g. for the production of small peptides) have a relatively clear genetic background, and intracellular interference signals from unwanted pathways have been minimally modified, which are different from the situation in traditional hosts. Based on these traits, media with defined nutrients can be designed to meet the requirements of growth and product generation based on the biosynthetic pathways embedded in chassis cells. Meanwhile, novel fermentation strategies based on intelligent online detection platforms with high precision and high efficiency have been employed to rationally control the fermentation process at both the extracellular and intracellular levels (Fig. 4). The key real-time parameters of a fermentation process have been calculated using online physiological data and used to guide the optimization of the fermentation process (Lu et al., 2016). Moreover, multiple component models of a fermentation process and the adaptive fuzzy control algorithm have been established using near-infrared spectroscopy (NIS; Landgrebe et al., 2010). With the advent of omics techniques, fermentation process control has become a more systematic and scientific method. For example, the advanced UPLC/Q-TOF-MS online detection technique enabled the construction of a global chemome that was used to analyse the main components of fermentation broths and target products (Paglia et al., 2012). Additionally, intracellular signals that reflect the metabolic states of chassis cells have also been accurately monitored and evaluated using next-generation analysis tools, such as Oxford Nanopore Technologies (ONT; Garalde et al., 2018). With the identification of intracellular signals, researchers have been able to quantitatively analyse and control key parameters (e.g. temperature, DO and pH), which usually exert marked effects on the fermentation process and control the metabolic states of chassis cells, via the online feedback system.

Concluding remarks

Small peptides, a group of bioactive compounds with low molecular weights and complex structures, have become an area of focus because of their therapeutic potential (Daliri et al., 2018). This review provides a good example of gaining insights into peptide compounds by a combination of chemical and cell biological approaches. Given the complex structures of bioactive peptides, the design and development of cell factories to synthesize these peptides might be a promising approach to bypass the complex chemical synthesis. Microbial production, over the past decade, has made it possible to obtain small peptides from natural isolation to laboratory preparation by engineering the biosynthetic pathway using advanced synthetic biology toolkits and emerging strategies. A part of sophisticated structures has also been realized through the enzymatic reactions within synthetic chassis cells, which further contribute to solving the availability and complexity issue of small peptides. The

![Fig. 4. Schematic illustration of fermentation process control based on the extracellular and intracellular levels.](image-url)
remained challenge is how to achieve large-scale production because the yield of small peptide cannot meet market requirements currently. Additionally, novel peptides and their analogs that might possess attractive pharmacological properties are required to be exploited in response to the risk of drug resistance. Recent researches showed that these new compounds can be synthesized by rational biosynthesis approaches, such as combinatorial biosynthesis (Yan et al., 2018). More efforts are still needed to elucidate the biosynthetic mechanisms of small peptides and explore synthetic approaches for accessing to new chemical structures. These state-of-the-art structures will further enrich the library of therapeutic candidates.

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Conflict of interest

The authors declare no competing interests.

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