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Research review paper

Synthetic biology and metabolic engineering of actinomycetes for natural product discovery

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ABSTRACT

Actinomycetes are one of the most valuable sources of natural products with industrial and medicinal importance. After more than half a century of exploitation, it has become increasingly challenging to find novel natural products with useful properties as the same known compounds are often repeatedly re-discovered when using traditional approaches. Modern genome mining approaches have led to the discovery of new biosynthetic gene clusters, thus indicating that actinomycetes still harbor a huge unexploited potential to produce novel natural products. In recent years, innovative synthetic biology and metabolic engineering tools have greatly accelerated the discovery of new natural products and the engineering of actinomycetes. In the first part of this review, we outline the successful application of metabolic engineering to optimize natural product production, focusing on the use of multi-omics data, genome-scale metabolic models, rational approaches to balance precursor pools, and the engineering of regulatory genes and regulatory elements. In the second part, we summarize the recent advances of synthetic biology for actinomycetal metabolic engineering including cluster assembly, cloning and expression, CRISPR/Cas9 technologies, and chassis strain development for natural product overproduction and discovery. Finally, we describe new advances in reprogramming biosynthetic pathways through polyketide synthase and non-ribosomal peptide synthetase engineering. These new developments are expected to revitalize discovery and development of new natural products with medicinal and other industrial applications.

1. Introduction

Natural products (NP) are bioactive molecules that are used for many different purposes such as herbicides, chemotherapeutics, immunosuppressants, and antibiotics (Berdy, 2005; Berdy, 2012; Hwang et al., 2014). For decades, *Streptomyces,* Gram-positive bacteria belonging to the Actinobacteria phylum, represented one of the main sources of novel NPs that are medically and industrially relevant (Bibb,

Abbreviations: NPs, natural products; antiSMASH, antibiotics and secondary metabolites analysis shell; BGC, biosynthetic gene cluster; 2D-DIGE, 2D-differential in gel electrophoresis; LC-ESI-LIT-MS/MS, nano-liquid chromatography electrospray linear ion trap tandem mass spectrometry; LC-MS/MS, liquid chromatography–coupled tandem MS; GEM, genome-scale metabolic network model; GC-MS, gas-mass spectrometry; PKs, polyketides; PPP, pentose phosphate pathway; BCDH, branched chain α-keto acid dehydrogenase; MSGE, multiplexed site-specific genome engineering; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9; ICE, *in vitro* CRISPR/Cas9-mediated editing; PII, pristinamycin II; TFRs, TetR family regulators; Lrps, leucine-responsive regulatory proteins; BCAA, branched-chain amino acid; PI, pristinamycin I; GBL, γ-butyrolactone-type; RBS, ribosome binding site; 5'UTR, untranslated regions; TAR, transformation-associated recombination cloning method; mCRISTAR, multiplexed CRISPR/Cas9- and TAR; NRPSs, nonribosomal peptide synthetases; PKS/NRPS, hybrid polyketide synthase non-ribosomal peptide synthetases; mmr, methylenomycin; hyg, hygromycin; BAC, bacterial artificial chromosomes; PAC, P1-derived artificial chromosome; LCHR, linear plus circular homologous recombination; LLHR, linear-plus-linear homologous recombination; CATCH, Cas9 associated targeting of chromosome segments; CISMR, CRISPR-mediated isolation of specific megabase-sized regions of the genome; PFGE, pulsed-field gel electrophoresis; DiPaC, direct pathway cloning; crRNA, CRISPR RNA; tracrRNA, Cas9trans-activating crRNA; PAMs, trinucleotide protospacer adjacent motifs; sgRNA, single guide RNA; HDR, homology-directed repair; NHEJ, nonhomologous end joining pathway; TX-TL, transcription-translation systems; AT, acyltransferase domain; DEBS, 6-deoxyerythronolide synthase; A, adenylation; C, condensation; T, thiolation; XUs, exchange units; KS, keto-synthase; CP, carrier protein.

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2013). Approximately 50% of the currently used antimicrobial drugs were discovered during the golden age of antibiotic discovery, from 1950 to 1960, using a traditional strain screening and fermentation approach. However, after this prolific time, antibacterial discovery has seen a dramatic decline. In the last twenty years, only about 30 new drugs were approved, of which only five belong to novel structural classes (Butler et al., 2017).

Advances in genome sequencing and genome mining methods revealed the huge biosynthetic potential for production of unknown NPs. In fact, genome mining with specialized software like antiSMASH (Blin et al., 2017; Weber et al., 2015), showed that a single actinomycetal genome harbors around 30 clusters on average. As bacterial whole genome sequences can now be easily and cheaply obtained, genome mining for secondary metabolite biosynthetic gene clusters (BGCs) has become a routine method of NP discovery in actinomycetes (Ziemert et al., 2016) complementing the classical chemistry-driven screening (Blin et al., 2017).

Genetic manipulation of actinomycetes is very challenging due to their GC-rich genomes and morphological/physiological characteristics, specifically their filamentous morphology and slow growth (Lee et al., 2009). Hopwood and colleagues established the DNA transformation protocol for *Streptomyces* in 1978 (Bibb et al., 1978), beginning the development of genetic manipulation tools for actinomycetes. Further development of these tools has remained relatively slow compared to that of tools developed for industrial biotechnology model organisms like *E. coli* and yeast. However, metabolic engineering, through integration with systems biology and synthetic biology, has significantly contributed to the discovery and production of new NPs. Systems metabolic engineering strategies (Lee et al., 2011; Lee et al., 2012; Lee and Kim, 2015) advancing rapidly will help establish even more efficient platforms for engineering actinomycetes for producing valuable natural and non-natural products (Kim et al., 2016; Park et al., 2018).

The aim of this review is to summarize both the conventional and novel approaches of metabolic engineering, and synthetic biology applications in actinomycetes, providing general strategies for NP discovery and production.

2. Metabolic engineering tools to optimize natural product production

The general objective of metabolic engineering is to understand and engineer a cells metabolic network at a systems level (Lee and Kim, 2015). In recent years, various metabolic engineering strategies have been developed and integrated with other fields, such as systems biology and synthetic biology, to discover new BGCs and create industrial producer strains (Sagt, 2013). However, most systems metabolic engineering approaches have been carried out with a model organism like *E. coli* or *Saccharomyces cerevisiae*. Only in recent years have these technologies been applied to the optimization of NP biosynthesis in actinomycetes (Robertsen et al., 2018; Weber et al., 2015). In this section, general metabolic engineering strategies for the production of NPs by actinomycetes are discussed.

2.1. Use of multi-omics data

The development of multi-omics techniques, including genomics, transcriptomics, proteomics, and metabolomics, has enabled systems-level analysis of cell physiology, which allows the prediction of new engineering targets and/or the re-design of fermentation strategies (Figure 1) (Chae et al., 2017; Palazzotto and Weber, 2018). Transcriptional changes in gene expression levels have been extensively analyzed to unveil the molecular mechanisms controlling the metabolic switch and production of NPs (Liu et al., 2013; Nieselt et al., 2010). In particular, a transcriptomics based approach has been used to investigate the complex network of signaling and regulatory proteins controlling the morpho-physiological differentiation occurring during actinomycetes growth (Botas et al., 2018; Zhou et al., 2015).

A systematic investigation of the relationship between transcription and translation at a genome-wide scale in *S. coelicolor* A3(2) during different growth phases revealed that also translational control influences the expression of secondary metabolic genes in *Streptomyces coelicolor* A3(2) and that engineering the translational control systems could offer a novel strategy to enhance secondary metabolite production (Jeong et al., 2016). In this study, furthermore 3,570 transcription start sites and the identification of 230 small RNAs representing ~21% of leaderless mRNAs were characterized. The ribosome profiling analysis indicated that the translation efficiency of secondary metabolic genes is negatively correlated with transcription and is generally reduced after the transition growth phase.

Recently, an integrated system biology approach, using constraint-based metabolic modeling with time-resolved RNA-seq data, was adopted to characterize the *Streptomyces ambofaciens* metabolism and to identify potential metabolic engineering targets for the overproduction of the secondary metabolites (Fondi et al., 2017).

Proteomics offers the possibility of identifying genes encoding proteins that are positively or negatively associated with the activation of secondary metabolism by comparison of protein expression levels. By studying changes in protein abundance during the growth it is possible to investigate the regulation of biological processes and metabolic pathways involved in both biomass and antibiotic production. A differential proteomic analysis performed on *Amycolatopsis balhimycina* DSM5908 before and during balhimycin production provided insights into the major metabolic pathways/molecular processes associated with antibiotic production (Gallo et al., 2010). These results revealed that amino acids, amino sugars, and central carbon metabolism intermediates were upregulated during antibiotic production, which suggests that they are interesting targets for the construction of high-yielding producer strains. This tight relationship between primary metabolism and NP biosynthesis was investigated in the model actinomycete *S. coelicolor* A3(2) during its growth on minimal medium supplemented with the tryptophan amino acid (Palazzotto et al., 2015). The results of 2D-DIGE and LC-ESI-LIT-MS/MS analyses showed that tryptophan stimulates anabolic pathways and promotes the accumulation of key factors associated with morphological and physiological differentiation at the late stages of growth.

Proteomics analyses also allow the study of post-translational events associated with many different metabolic processes. A high-resolution mass spectrometry-based proteomics approach was used to investigate the acetylproteome of *Saccharopolyspora erythraea*, the producer of erythromycin (Huang et al., 2015). The results revealed that acetylated proteins are involved in protein synthesis, glycolysis/gluconeogenesis, the TCA cycle, fatty acid metabolism, secondary metabolism, and the feeder metabolic pathways of erythromycin synthesis. A combined omics approach has been applied to identify the rate-limiting steps for production of spinosad (a mixture of spinosyn A and spinosyn D) in the *Streptomyces* hosts *S. lividans* TK24 (C4I6-M) and *S. albus* J1074. Transcriptomics and proteomics analyses were used to analyze the expression of spinosyn biosynthetic genes in the heterologous host. The precursor supply and cofactor availability, as well as the biosynthetic intermediate of spinosyn, were investigated by metabolomics analysis. The omics results were used as a platform for rational refactoring of the heterologous biosynthetic pathway in *S. albus* leading to a 1000× increased production compared to the original heterologous expression experiment (Tan et al., 2017).

Another important component of the systems-wide analysis of antibiotics producers is metabolomics, which is a comprehensive analysis of the entire complement of small metabolites in a biological sample. Comparative metabolomics approaches have been successfully used to identify novel compounds in actinomycetes. By comparing the metabolic profiles of wild-type and mutant strain or by comparing strains grown in different conditions, it was possible to obtain information

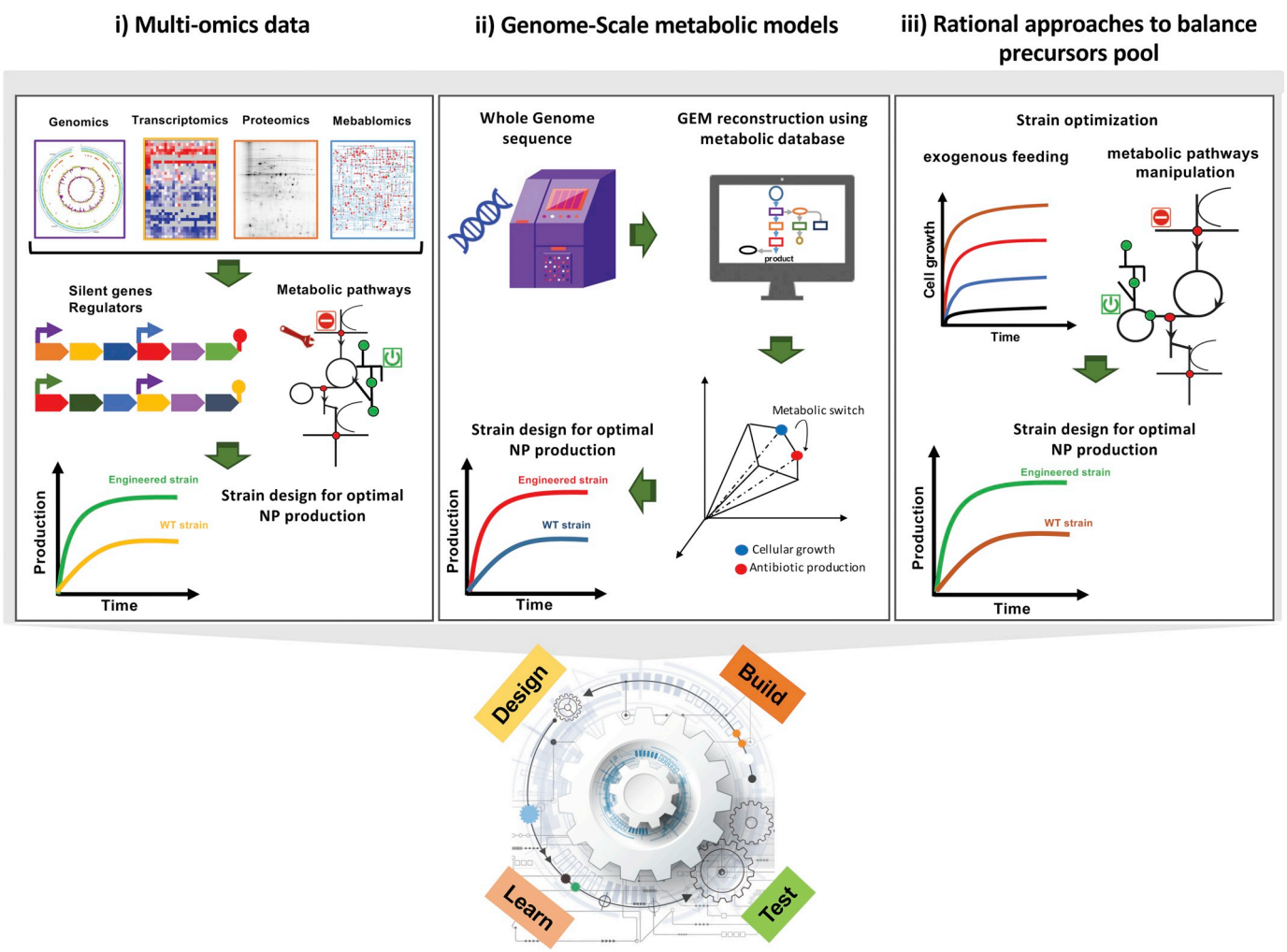

Fig. 1. Schematic representation of the metabolic engineering workflow based on a rational, predictive approach. Strategies for discovering new or silent biosynthetic gene clusters and natural compounds. The design–build–test–learn cycle of metabolic engineering. i) High-throughput analysis such as genomics, transcriptomics, proteomics, and metabolomics can be used to uncover the genes and compounds that can be manipulated to activate or increase NP production. ii) Construction of metabolic modeling by genomic-scale metabolic model analysis. The complete genome sequence and the results from high-throughput analysis are used as input to build models for precursor supply optimization, intracellular redox balance, and prediction of gene manipulation. iii) Rational approaches to optimize the metabolic flux within the target biosynthetic pathways through optimization of precursors supply or overexpression or deletion of target genes.

about differentially abundant molecules such as oasamycin and desertomycin (Covington et al., 2017; Doroghazi et al., 2014). The secreted metabolome of *Streptomyces chartreusis* NRRL 3882, which is the producer strain of calcimycin and tunicamycin (Doroghazi et al., 2011; Senges et al., 2018), was cultivated in different media and analyzed by untargeted data-dependent LC-MS/MS and molecular networking. Using this strategy, the set of metabolites produced in these specific growth conditions were identified, along with eight previously undescribed desferrioxamines. Metabolomics is also a powerful tool to study the influence of primary metabolites on secondary metabolite production. A comparative metabolomics analysis was performed to elucidate the effect of S-adenosylmethionine on the avermectin titer in *Streptomyces avermitilis* (Tian et al., 2017). GC–MS results showed an upregulation of key primary metabolic pathways: central carbon metabolism, amino acid metabolism, and fatty acid metabolism, and thus an increased availability of precursors for avermectin biosynthesis.

2.2. Genome-scale metabolic models

Genome-scale metabolic network models (GEM) are important tools for rational metabolic engineering of cell factories. Various algorithms have been developed to predict global metabolic flux distributions under given genetic and environmental conditions. Due to the increasing number of complete genome sequences available for organisms and the advances in high-throughput technologies (Lee et al., 2012), modeling approaches have contributed to the optimization of precursor supply, intracellular redox balances, and gene manipulation predictions (knockouts and over-expression), allowing maximization of the production yield of a target product (Kim et al., 2015; Kim et al., 2017; Wang et al., 2018).

In particular, the use of GEM to study the interactions between primary and secondary metabolism at system level has opened the possibility to predict gene manipulation targets to redirect primary metabolic fluxes toward the production of the desired NP. To date, twelve actinomycetes GEMs were reconstructed (Mohite et al., 2018) which serve as a basis for identifying engineering targets to improve secondary metabolite production.

In order to improve the production of the glycopeptide balhimycin, a GEM of *A. balhimycina* was generated from genome sequencing and annotation data (Vongsangnak et al., 2012). This model was used to simulate and predict the phenotypic characteristics of *A. balhimycina* and to identify metabolic reactions and targets for metabolic engineering.

A GEM of *S. erythraea* was manually constructed and used to assess

the potential benefit of amino acid supplementation to erythromycin production (Licona-Cassani et al., 2012). In this study, the supplementation of five amino acids (compatible) with erythromycin production induced a 50% increase in erythromycin production. Similar results were obtained in *Saccharopolyspora spinosa*. Simulations of the GEM indicated that the amino acid supply is likely a bottleneck in spinosad production (Wang et al., 2014). The model was validated experimentally, leading to spinosad production that was 86.5% higher than the parent strain. The GEM approach has also been applied to improve the titer of other NPs. The yield of rapamycin in *Streptomyces hygroscopicus* was increased by 142.3% (Dang et al., 2017). The yield of FK506 in *Streptomyces tsukubaensis* was increased by 161% (Huang et al., 2013; Wang et al., 2017). Clavulanic acid production in *Streptomyces clavuligerus* was also improved by use of the GEM approach (Toro et al., 2018).

2.3. Rational approaches to balance precursor pools

A key factor in the production of a target compound is the availability of biosynthetic precursors. They are generally produced through the catabolism of different carbon sources such as monosaccharides, fatty acids, and amino acids. The intracellular level of those metabolites can be controlled by different rational approaches such as exogenous feeding and genetic manipulation of bottleneck enzymes (Figure 1).

Different studies demonstrated that exogenous feeding with precursors can indeed increase the production of polyketides, a large family of NPs built from acyl-CoA monomers (Chan et al., 2009; Mo et al., 2009). The production of these very diversified molecules resembles fatty acid biosynthesis: in polyketide biosynthesis, malonate-derived extender unit are attached to the phosphopantethein prosthetic group of an acyl carrier protein, linked together by Claisen condensations and, subsequently, oxidatively processed. Due to the fact that fatty acids and PKs share common precursor pools, flux control between PK and fatty acid biosynthesis is a key strategy to enhance PK production (Coze et al., 2013).

In *S. coelicolor* A3(2), the production of the type II PK actinorhodin was induced by increased levels of the precursor acetyl-CoA, derived from glucose, and amino acid degradation pathways (Palazzotto et al., 2015; Stirrett et al., 2009). Similar results were observed in *S. tsukubaensis*, in which the production of tacrolimus was optimized by evaluating the intracellular response to enhancement of malonate, lysine, shikimate, and citrate synthetic precursor pathways in tacrolimus biosynthesis (Wang et al., 2017). A GEM was used to screen all possible limiting steps and predict targets for strain engineering.

Even without the availability of GEMs, genetic manipulation of potential bottleneck enzymes of primary metabolic pathways represents an alternative strategy of increasing the intracellular pool of precursors and to redirect the flux towards the biosynthesis of the desired metabolite (Olano et al., 2008). Successful manipulation of the central carbohydrate metabolism has been reported for the increased production of several PKs such as erythromycin, oligomycin, monensin B, and actinorhodin (Borodina et al., 2008; Reeves et al., 2007; Ryu et al., 2006). In *S. coelicolor* A3(2), actinorhodin production was increased through the reduction of the carbon flux into either the PPP or glycogen synthetic pathways. This reduction was achieved by deletion of the phosphofructokinase gene *pfkA*, along with the glucose-6-phosphate dehydrogenase genes *zwf1* or *zwf2*, and overexpression of an acetyl coenzyme A carboxylase (Ryu et al., 2006). In *Streptomyces venezuelae* ATCC 15439, pikromycin production was improved by the manipulation of three key enzymes of branched-chain amino acid catabolism, 3-ketoacyl acyl carrier protein synthase III, acyl-CoA dehydrogenase, and BCDH (Yi et al., 2018). Pikromycin production was further increased by overexpression of the methylmalonyl-CoA mutase that direct the products of BCDH reactions away from fatty acid synthesis and toward the synthesis of acetyl-CoA, propionyl-CoA, and methylmalonyl-CoA (Zhang et al., 1999). In *A. balhimycina*, the producer strain of the glycopeptide balhimycin, the deep relationship between primary metabolism and balhimycin production was investigated through the analysis of two genes, 3-deoxy-D-arabino-heptulosonate 7-phosphate (*dahp*) and prephenate dehydrogenase (*pdh*), both contained in the balhimycin biosynthetic cluster. The *dahp* and *pdh* genes, which encode two key enzymes in the shikimate pathway, were overexpressed both individually and together. The resulting mutants were characterized for glycopeptide productivities, biomass yields, and growth rates, thus revealing that the strain carrying an extra copy of both *dahp* and *pdh* genes had improved glycopeptide production by a factor of approximately three (Thykaer et al., 2010). In *Streptomyces argillaceus* the production of mithramycin, a polyketide with antitumor activity (Remsing et al., 2003), was improved by increasing the intracellular pool of glucose-1-phosphate and/or malonyl-CoA precursors. This was achieved by overexpressing the phosphoglucomutase gene or the acetyl-CoA carboxylase *ovmGIH* genes in *S. argillaceus,* or by inactivation of the acyl-CoA:diacylglycerol acyltransferase gene *aftAa* (Zabala et al., 2013). Another interesting example is the engineering of *Streptomyces kanamyceticus* to modulate the biosynthesis of kanamycin A and B. In order to obtain an optimal kanamycin A-producing strain, the *kanJ* and *kanK* genes, which encode two enzymes responsible for converting kanamycin B to kanamycin A, were overexpressed (Gao et al., 2017).

2.4. CRISPR/Cas9

In most archaea and bacteria, CRISPR/Cas is an adaptive immune system for defense against exogenous DNA or RNA (Barrangou et al., 2007). Especially the type II CRISPR/Cas9 systems of *Streptococcus pyogenees* is widely used for biotechnological applications. It consists of three elements: (1) the endonuclease Cas9; (2) crRNA, a short RNAs conferring target site specificity and, (3) tracrRNA, a short RNA facilitating the crRNA processing and recruitment. (Chylinski et al., 2014; Koonin et al., 2017). The crRNA-tracrRNA duplex form a complex with Cas9 and scans the target genome for the presence of PAMs in which the 5' sequence is complementary to the spacer sequence in the crRNA-tracrRNA duplex. Once the complex binds this specific target sequence, the Cas9 nuclease activity is triggered (Nishimasu et al., 2014). In the simplified system of the native CRISPR, the crRNA and the tracrRNA are fused into a single synthetic guide RNA (sgRNA) (Mali et al., 2013).

Due to the precise and controllable action of Cas9 on target DNA sequences, CRISPR/Cas9 has also become an excellent tool for high-efficiency genome editing and specific refactoring of BGCs and other large DNA. CRISPR/Cas9-derived tool allow for gene manipulation, deletion, insertion, site direct mutagenesis in *Streptomyces* and also many genetically recalcitrant organisms (Huang et al., 2015; Kim et al., 2017; Tong et al., 2018).

In 2015, a CRISPR/Cas system for rapid multiplex genome editing of *Streptomyces* called pCRISPomyces was developed (Cobb et al., 2015) by Zhao and co-workers. This method represented an alternative to the laborious and time intensive protocol for gene deletion in *Streptomyces*. pCRISPomyces-1 contains both crRNA and tracrRNA, along with *cas9*, and pCRISPomyces-2 uses sgRNA instead of tracr/crRNA. In both constructs, the expression of CRISPR/Cas elements is guided by a strong promoter. The efficiency of pCRISPomyces as a genome editing tool was demonstrated by targeted chromosomal deletions in different gene clusters in various *Streptomyces*.

Most of the CRISPR-Cas9 applications use the host homologous recombination machinery to realize both targeted deletion and insertion. In addition to these CRISPR-Cas9 systems, Tong and co-workers used nonhomologous end-joining (NHEJ) repair to introduce deletions or frame- shifts (Tong et al., 2015).

An extension of CRISPR/Cas9 technology was recently developed to perform strategic promoter knock-in for the activation of silent BGCs in native *Streptomyces* hosts (Zhang et al., 2017). Thanks to this method, a novel doubly glycosylated 24-membered polyene macrolactam, auroramycin, was discovered in *S. roseosporus* by inserting the *kasO**

promoter to constitutively express the transcriptional activator of the auroramycin BGC (Lim et al., 2018).

In addition to *in vivo* applications, CRIPSR/Cas9 can also be used *in vitro*. In 2015, a highly efficient *in vitro* CRISPR/Cas9-mediated editing (ICE) system was developed exploiting CRISPR/Cas9 as an *in vitro* programmable endonuclease to edit actinomycete BGCs while avoiding non-specific recombination (Liu et al., 2015).

2.5. Multiplexed site-specific genome engineering (MSGE)

MSGE is a general metabolic engineering approach allowing a single step multi-copy chromosomal integration of a target BGC by the "one integrase-multiple *attB* sites" concept (Li et al., 2017). Using CRISPR/Cas9, multiple artificial *attB* sites (including three artificial *Φ*C31 sites and two artificial *Φ*BT1 *attB* sites) were inserted into the *Streptomyces pristinaespiralis* genome. These insertions stably integrated multiple copies of the pristinamycin II (PII) BGC, reaching the highest reported PII production in flask and batch fermentations. The validity of this method was also tested in *S. coelicolor* A3(2), in which up to four copies of the chloramphenicol or YM-216391 (an anti-tumor compound) BGCs were inserted in a single step.

2.6. Regulatory gene and regulatory sequence manipulation

The expression of NP BGCs is governed by very complex regulatory networks, which in most cases are not well studied or understood. This constitutes a major bottleneck for optimizing these pathways. The engineering of regulatory elements such as promoters, ribosome binding sites (RBSs), and terminators, tries to overcome the complex cellular regulatory network and tune gene expression. In this section we describe different strategies to manipulate regulatory genes and/or other regulatory modules to activate specific pathway or achieve an optimal NP production in both the native and/or heterologous host.

2.6.1. Regulatory genes

Among the various families of transcriptional regulators, the TetR family of regulators (TFRs) is widely distributed in bacteria and represents one of the largest groups in *Streptomyces*. The complex interaction between members of this family regulates important cellular processes such as osmotic stress, catabolic pathways, differentiation, and expression of many NP BGCs. Genes encoding TetR-like repressor have been identified in many streptomycetes within or in the proximity of secondary metabolite gene clusters. This suggests that they have a role in the regulation of gene cluster expression (Ahn et al., 2012). Most of the family members act as homodimeric transcriptional repressors, a small number also have a function of activators or dual repressors/activators. In *S. avermitilis*, several TetR regulators have been characterized as negative or positive regulators of avermectin production. AveT activates both avermectin production and morphological differentiation by affecting transcription of the cluster-encoded activator gene *aveR*. The overexpression of *aveT* in *S. avermitilis* wild-type and industrial strains led to a 22% increase of avermectin production (Liu et al., 2015). Similar results were obtained in the erythromycin high-producing strain *S. erythraea* WB. Here, the overexpression of the TetR regulator SACE_7301 increased the erythromycin A titers by 27% in both wild-type *S. erythraea* A226 and high-yield industrial strain WB by increasing the transcription of erythromycin biosynthetic genes (Wu et al., 2014). In another report, erythromycin production was enhanced by 41% in the industrial strain *S. erythraea* WB by engineering the SACE_5388 protein, a transcriptional regulator belonging to the leucine-responsive regulatory proteins (Lrps) family, which regulates the transcription of the *SACE_5387-5386* operon. This operon codes for a branched-chain amino acid ABC transporter (Liu et al., 2017). Erythromycin biosynthesis requires propionyl-CoA and methylmalonyl-CoA, which are products of branched-chain amino acid (BCAA) degradation pathways. The results indicated that the deletion of the Lrp, combined with overexpression of the target *SACE_5387-5386* increased the BCAAs transport and catabolism, thus providing more metabolite precursors for erythromycin biosynthesis.

In another study, Salas and co-workers were able to activate six silent/cryptic gene clusters in *S. albus* J1074, that had been previously identified using genome-mining approaches, with different approaches: insertion of a strong constitutive promoter (ermE*p), overexpression of the positive regulatory gene *sshg_05706*, and heterologous expression of the LuxR-family activator *pimM* of the pimaricin cluster (Olano et al., 2014).

A combinatorial metabolic engineering strategy was used for improving pristinamycin antibiotic production in *Streptomyces pristinaespiralis*. Pristinamycin is a streptogramin antibiotic containing two chemically unrelated compounds, pristinamycin I (PI) and PII, which show strong synergistic effects. The compounds are synthesized by a NRPS and a hybrid PKS/NRPS assembly line, respectively (Mast et al., 2011; Mast et al., 2011). The expression of the PI/PKII BGCs is controlled by a complex regulatory cascade. In order to overcome toxic effects of high titers of the synergistically active PI/PII combination on *S. pristinaespiralis* growth (Li et al., 2015), a strain producing high titer of only pristinamycin I, was generated by Lu and co-worker by deletion of *papR3* repressor gene and the integration of an extra copy of PI BGC in the chromosome (Meng et al., 2017). A similar approach was used to generate a high PII producer strain. An extra copy of PII BGC was introduced and all seven regulatory genes were manipulated. Higher PII titers were obtained when one of the two repressor genes, *papR3* or *papR5,* was deleted in combination with overexpression of both activator genes, *papR4* and *papR6*.

Another strategy adopted to increase antibiotic production consists of the gene manipulation of γ- butyrolactone (GBL) receptor genes. GBL-type molecules are a well-characterized family of signaling molecules that regulate antibiotic biosynthesis and morphological differentiation in *Streptomyces* species through regulatory cascades (Du et al., 2011; Takano, 2006). The best-studied GBL is the so-called A-factor (2-isocapryloyl-3R-hydroxymethyl-gamma-butyrolactone) of *Streptomyces griseus* (Horinouchi and Beppu, 1992). During the early stage of growth, the A-factor receptor ArpA blocks the transcription of the *adpA* gene (an AraC/XylS family transcriptional activator gene) by binding to the promoter region. When the concentration of A-factor reaches a critical threshold, the A-factor/ArpA complex dissociates from the *adpA* promoter resulting in the activation of *adpA* transcription and consequent activation of genes involved in NP biosynthesis, such as streptomycin (Ohnishi et al., 2005). GBL also play important roles in other species. By tandem deletion of GBL receptor genes, the validamycin production of *S. hygroscopicus* was increased by 55% (Tan et al., 2015). The results showed that the transcription level of *adpA-H*, the *adpA* orthologue, controlling the transcription of the validamycin *valABC* and *valKLMN*-genes was enhanced after deletion of *arpA*. In addition, it was demonstrated that the *arpA* deletion to the overproduction of validamycin not only in a wild-type (*S. hygroscopicus* 5008), but also in a high-producing industrial strain (*S. hygroscopicus* TL01).

2.6.2. Promoters

Promoter engineering is a widely used strategy to trigger the expression of silent BGCs in bacterial genomes. By replacing native regulatory sequences with well-characterized constitutive or inducible promoters, it is possible to bypass the complex BGC transcriptional regulation thus inducing the expression of biosynthetic genes (Figure 2).

A variety of constitutive and inducible elements are currently available to finely control gene expression levels for streptomycetes (Myronovskyi and Luzhetskyy, 2016). Commonly used constitutive promoters to activate gene expression are: the promoters of the erythromycin resistance gene ermE of *S. erythraea*, ermEp1, ermEp1*, ermEp2, ermEp, and ermE*p (Bibb et al., 1985; Bibb et al., 1994; Schmitt-John and Engels, 1992; Takano et al., 1995); the phage I19

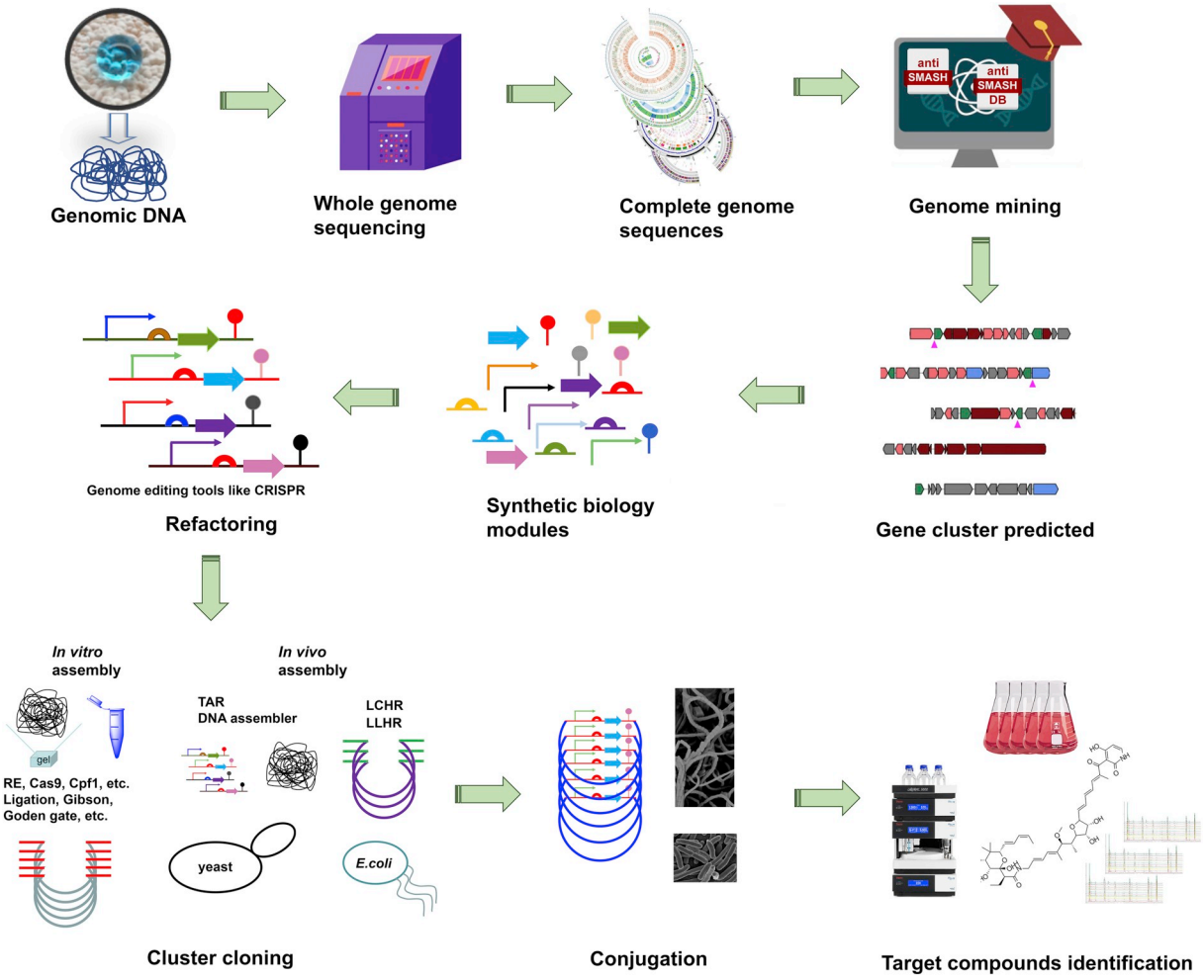

Fig. 2. Synthetic biology workflow. Summary of the current synthetic biology strategies and tools for the production of natural products. Genome sequencing using next-generation genome sequencers, and bioinformatics tools such as antiSMASH to mine biosynthetic gene clusters in genomic databases. Optimization of synthetic regulatory elements, promoters, ribosome binding sites (RBSs) and terminators to bypass the complex cellular regulatory network controlling BGCs expression. Use of CRISPR as genome editing tool for gene cluster refactoring. Direct cloning and reassembly of secondary metabolite BGCs using *in vitro* and *in vivo* methods.

originated promoter SF14p (Labes et al., 1997); and the kasOP and kasOP* promoters (Wang et al., 2013). Inducible promoters often used are: the thiostrepton-inducible promoter PtipA (Murakami et al., 1989), requiring the TipAL protein; the synthetic tetracycline-inducible promoter tcp830 (Rodriguez-Garcia et al., 2005); and synthetic resorcinol-inducible and cumate-inducible promoters (Horbal et al., 2014). Due to the limited number of well-characterized promoters in actinomycetes, a library of synthetic promoters was constructed and characterized, using as reporter genes *gusA*, *gfp*, *aphII*, and *rppA*. The library was generated through randomization of -10 and -35 sequences of the ermEp1 promoter, with strengths ranging from 2% to 319% compared to ermEp1. RNA sequencing data were used to compare synthetic promoter strength to described promoters like tcp and ermEp in *S. albus* J1074, *Saccharothrix espanaensis* DSM 44229, and *Salinispora tropica* CNB- 440 (Siegl et al., 2013). A synthetic promoter library was also used to modulate the expression level of the positive regulator *actII*ORF4, which controls the transcriptional activation of the actinorhodin BGC in *S. coelicolor* A3(2) (Sohoni et al., 2014). The actinorhodin production of the *S. coelicolor* A3(2) expression library was compared to *S. coelicolor* A3(2) wild-type and *S. coelicolor* A3(2) with *actII*ORF4 under the control of the strong constitutive promoter ermE*p. The results showed higher actinorhodin yield and final titer in one of the strains of the library compared to the control. Interestingly, the transcriptional data showed that despite the expression being weaker than in the control, a higher actinorodin production was obtained due to the activation in an earlier stage of growth compared to the wild type. In order to provide strong promoters for NP production in the chassis organism *Streptomyces* sp. FR-008, Liu and co-worker chose and evaluated 20 strong promoters on the base of GusA activity in FR-008 (Liu et al., 2016). The selected promoters played different regulatory roles and, compared with ermE*p, the strength of these promoters comprise a library with a constitutive range of 60–860%. The results obtained in this work provided useful biological elements for genetic engineering and expression of exogenous genes in FR-008.

Zhao and co-workers explored native constitutive promoters in *S. albus* J1074 by RNA-seq analysis (Luo et al., 2015). A total of 32 promoters were identified and characterized using *xylE* as a reporter gene. Among those, ten showed stronger activity than the ermE promoter in *S. albus*. Recently, an inducible expression system using xylose sugar as inducer was developed to regulate protein expression in *Streptomyces* (Noguchi et al., 2018). The system, consisting of a vector containing *S. avermitilis* XylR, *xylA* promoter, and *xyl* operon, was tested in *Streptomyces lividans*. A strict regulation of the reporter gene expression and activity was observed in *S. lividans*. The results strongly suggested the applicability of the xylose-induced system to controlling the heterologous expression of proteins and toxic products in *Streptomyces* strains.

Even though the substitution of native promoters has been widely used as a general strategy to activate and/or increase BGC transcription, this approach often does not directly lead to the overproduction of the desired metabolite. In fact, negative side effects on cell fitness and low

titer of the desired product are commonly observed (Bienick et al., 2014; Pitera et al., 2007). Several studies highlighted that, in order to improve the production of a target metabolite, it is crucial to balance the expression genes encoding bottleneck enzymes in the biosynthetic pathway (Bruheim et al., 2002; Coze et al., 2013). For designing an efficient gene expression system, relying only on the measurement of mRNA transcript levels is not enough (Yi et al., 2017). The amount of protein synthesized depends on the efficiency of mRNA translation, which itself depends on the mRNA secondary structure and the strength of the ribosome binding site (RBS). Yi et al. proposed a novel method for screening and designing pairs of promoters and 5'UTR regions with constant strength that considers both transcription and translation. These pairs of promoters and regions were selected by using multi-omics datasets from *S. coelicolor* A3(2), including RNA-seq, Ribo-seq, and TSS-seq.

In 2015, a universal toolbox of synthetic modular regulatory elements was developed for activating the expression of cryptic gene clusters at various levels by replacing native promoter and RBS sequences. The efficiency of this approach was confirmed by the overproduction of a cryptic lycopene BGC in *S. avermitilis* (Bai et al., 2015). The ability of RNA-guided endonuclease Cas9 to introduce targeted double strand breaks into a DNA sequence, combined with the TAR system, was used to develop an efficient method for multiplex promoter engineering of silent BGCs called yeast-based promoter engineering platform (mCRISTAR) (Kang et al., 2016). This method was applied to tetarimycin, lazarimide, and AB1210 gene clusters promoter refactoring (Kang et al., 2016). However, due to the lack of a robust set of orthogonal regulatory sequences, the use of the mCRISTAR system to activate the expression of cryptic BGCs has been limited. To overcome the unexpected formation of secondary structures that can possibly cause the cross-recombination between very similar regulatory sequences inserted into a single gene cluster, a library of constitutively active, synthetic *Streptomyces* regulatory sequences with the highest sequence divergence was generated (Ji et al., 2018). The library was constructed by complete randomization of regulatory sequences including both promoter and RBS regions. As a screening system the small indigoidine NRPS was used. Based on the amount of indigoidine produced, the regulatory sequences were grouped into strong, medium, and weak. Finally, the library was tested using the actinorhodin biosynthetic gene cluster as a model cluster.

Recently, Luzhetskyy and co-workers described an approach for cluster cloning refactoring based on the construction of a library of clusters of interest, consisting of the substitution of the native promoter with randomly generated constitutive synthetic promoters, and expression of the generated library in an appropriate host. With this strategy they were able to optimize the titers of bottromycin, a promising macrocyclic peptide, which finally allowed them to characterize new low-abundant derivatives that had not previously been described (Horbal et al., 2018).

2.6.3. Regulatory devices

Cellular controlling elements can be used to construct controllable programmable gene circuits responding to different extra- and intracellular inputs.

A very promising tool that does not rely on additional elements is use of regulatory RNA, specifically synthetic riboswitches (Lynch et al., 2007). Riboswitches are mostly located within the 5'UTR of an mRNA, and their expression is controlled by the highly specific binding of the aptamer domain (riboswitch binding region) with a ligand. A set of synthetic riboswitches have been engineered to recognize and bind cell permeable small molecules that are not metabolized by the cell (Topp and Gallivan, 2008; Wittmann and Suess, 2012). A method based on the theophylline-dependent synthetic riboswitches was established to control the gene expression in a temporally defined and dose-dependent manner in *S. coelicolor* A3(2). By cloning different synthetic theophylline-responsive riboswitches in front of a ß- glucuronidase reporter gene (*gusA*) and an endogenous agarase gene, the authors showed a dose-dependent activation of both reporter genes and the orthogonality of riboswitch regulation (Rudolph et al., 2013; Rudolph et al., 2015).

Due to the need of a non-leaky inducible system controlling the gene expression at both transcriptional and translational level, Horbal and Luzhetskyy developed a series of dual control regulatory devices in *Actinobacteria*, completely silent in the absence of inputs and, at the same time, highly inducible (Horbal and Luzhetskyy, 2016). These regulatory devices are based on cumate switches or resorcinol switches, which regulate the gene expression at the transcriptional level (Horbal et al., 2014), the theophylline riboswitch (Rudolph et al., 2013), or the hammerhead ribozyme that instead controls translation initiation (Horbal et al., 2014). In addition, a library of cumate-inducible promoters with a wide range of strengths was constructed. The authors showed the expression of reporter genes in different operons by using different concentrations of the same inducer and different promoters.

2.6.4. Terminators

Together with promoter sequences, terminators are essential elements in regulating the level at which the gene is transcribed. Differential expression of genes in the same operon can be achieved by weak terminators within an operon (Figure 2) (Deng et al., 1987). Unlike promoters, terminators were not extensively characterized in *Streptomyces*. Only a few Rho-independent terminators were experimentally studied, such as the phage derived terminators fd, t0, and Φ29; the *Streptomyces* methylenomycin (mmr) and hygromycin (hyg) terminators (Kieser et al., 2000); and recently the tt_{sbiB} terminator from *Mycobacteria,* which was reported to also be effective in *Streptomyces* (Horbal et al., 2018).

2.6.5. Ribosomal binding sites (RBSs)

In addition to regulation on the transcriptional level, translation initiation and translation efficiency also strongly affect protein yield (Figure 2) (Kaberdin and Bläsi, 2006). In the light of the need to control the gene expression at post-transcriptional or translational levels, Luzhetskyy and co-workers analyzed the influence of sequence changing of 5'UTRs on translation rate in *S. lividans* TK24. For the first time, it was demonstrated in Actinobacteria that RBSs can strongly affect translation efficiency, thus reducing the expression efficiency of a gene to zero in certain cases. A panel of 70 RBSs with different translation activities was characterized (Horbal et al., 2018) and an *in vivo* RBS-selector device was developed for the identification and selection of an optimal RBS for any gene of interest.

3. Metabolic engineering and synthetic biology strategy for the production and optimization of NPs in actinomycetes

3.1. Cluster assembly, cloning, and expression

Besides engineering the native host strains as described above, cloning the BGCs of interest and expressing them heterologously in a well-characterized host has emerged as an alternative strategy for BGC activation and new NP discovery (Nepal and Wang, 2018). Heterologous expression of BGCs in a genetically manipulatable host allows production and modification of the NPs biosynthetic pathways, bypassing the limitation associated with the genetic manipulation of native strains with methods like promoter refactoring, pathway-specific/global regulator manipulation, ribosome engineering, and other rational modifications (Figure 2) (Nah et al., 2017; Ren et al., 2017). Although there have been numerous advances in cloning systems and vector construction in recent years, cloning large-sized DNA harboring entire BGCs with repetitive sequences remains a challenging task (Tao et al., 2018). Nevertheless, several methods have been established both in *in vivo* and *in vitro* to clone and assemble large DNA fragments harboring BGCs (Tocchetti et al., 2018). In this context, we will describe the most recent and relevant methodologies developed for efficient BGC

Table 1
Examples of heterologously expressed BGCs using large DNA fragment cloning methods.

Heterologous host	Heterologous expression method	Original host	BGC size (kb)	BCG encoded compound	BGC cloning method	References
S. ambofaciens *S. roseosporus*	Integrative vector	*S. fradiae* NRRL 18160	60	A54145 (NRPS)	BAC	(Alexander et al., 2010)
S. avermitilis SUKA17	Integrative vector	*S. olivoriridis* OM13	70	Thioriridamide (Ribosomal peptide)	BAC	(Izumikawa et al., 2015)
S. albus J1074	Integrative vector	*S. versipellis 4083*	108	Versipelostatin (PKS I)	BAC	(Hashimoto et al., 2015)
S. lividans TK64	Integrative vector	*S. roseosporus* NRRL 11379	128	Daptomycin (NRPS)	BAC	(Miao et al., 2005)
S. coelicolor M1152	Integrative vector	*S. leeuwenhoekii*	80.2	Chaxamycin (PKS I)	PAC	(Castro et al., 2015)
S. coelicolor M1146	Integrative vector	*S. tsukubaensis*	83.5	FK506 (PKS I)	PAC	(Jones et al., 2013)
S. coelicolor M1146	Integrative vector	*S.* sp. T676	53.2	Anthracimycin (PKS I)	PAC	(Alt and Wilkinson, 2015)
S. lividans TK24 *S. lividans* K4-114	Integrative vector	*S.* sp. NRRL 30748	90	Meridamycin (PKS-NRPS)	pSBAC	(Liu et al., 2009)
S. coelicolor M145 *S. lividans* TK21	Integrative vector	*S.* sp. CK4412	80	Tautomycetin (PKS I)	pSBAC	(Nah et al., 2015)
Streptomyces 4F	Integrative vector	*S. coelicolor* A3(2)	33	Actinorhodin (PKS II)	LLHR	(Chen and Qin, 2011)
S. fradiae	Integrative vector	*S. bikiniensis*	80	Chalcomycin (PKS I)	LLHR	(Ward et al., 2004)
S. lividans TK24	Integrative vector	*S. chartreusis* AN1542	54.5	Complestatin (Glycopeptide)	LLHR	(Park et al., 2016)
S. coelicolor M512 *S. lividans* TK24	Replicative & Integrative vector	*S. pulveraceus* ATCC31906	48.6	Fostriecin PKS (PKS I)	LLHR	(Su et al., 2015)
S. coelicolor A3(2)	Integrative vector	*S. albus* DSM41398	106	Salinomycin (PKS I)	LLHR	(Yin et al., 2015)
S. coelicolor CH999	Replicative & Integrative vector	*Sorangium cellulosum* SHP44	56	Epothilone (PKS-NRPS)	LLHR	(Tang et al., 2000)
S. parvulus ATCC12434	Replicative vector	*S. coelicolor* M145	38	Undecylprodigiosin (NRPS)	LLHR	(Malpartida et al., 1990)
S. lividans K4-114 *S. lividans* K4-155	Replicative vector	*S. antibiticus*	65	Oleandomycin (PKS I)	LLHR	(Shah et al., 2000)
S. albus J1074	Integrative vector	*S.* sp. KCCM11116P	120	FK506 (PKS I)	LCHR	(Chen et al., 2014)
S. coelicolor M512	Integrative vector	*S. lusitanus* SCSIO LR32	37	Grincamycin (PKS II)	LCHR	(Zhang et al., 2013)
S. coelicolor M512	Integrative vector	*S.* sp. CNR-698	35	Ammosamides A-C (Alkaloid)	TAR	(Jordan and Moore, 2016)
S. albus J1074	Integrative vector	*S.* sp. Acta 1362	36	Grecocycline (PKS II)	TAR	(Bilyk et al., 2016)
S. lividans JT46	Integrative vector	*S.* sp. Tü6176	44.1	Nataxazole (PKS I)	TAR	(Cano-Prieto et al., 2015)
S. coelicolor	Integrative vector	*Saccharomonospora* sp. CNQ-490	67	Taromycin A (NRPS)	TAR	(Yamanaka et al., 2014)
S. lividans pZR94	Integrative vector	*S. sparsogenes* ATCC 25498	30	Sparsomycin (NRPS)	TAR	(Rui et al., 2015)

cloning and assembly in actinomycetes (Table 1).

The construction of Bacterial Artificial Chromosomes (BAC) shuttled between *E. coli* and *Streptomyces* hosts (Sosio et al., 2000) provided a system to clone large-size BGCs which could be stably maintained in a host amenable to genetic manipulation (Alduina and Gallo, 2012). Since the first description of *E. coli-Streptomyces* shuttle BAC vectors, several improved variants have been developed in the last decade, such as pSTREPTOBAC V, pSBAC, and pESAC13. Due to the BAC ability to carry DNA inserts up to 100 kb, BAC libraries were used to capture large BGCs such as the giant daptomycin BGC (128 kb) from *S. roseosporus* NRRL11379 (Miao et al., 2005). pStreptoBAC V was also used for cloning and heterologous expression of the 65 kb iso-migrastatin BGC (Feng et al., 2009), and the versipelostatin BGC from *Streptomyces versipellis* (Hashimoto et al., 2015). The pSBAC vector, which is able to switch replication from single-copy to high-copy in *E. coli*, was successfully used to clone and heterologously express the meridamycin BGC (90 kb) from *Streptomyces* sp. NRRL 30748 in *S. lividans* K4-114 (Liu et al., 2009). In 2015, pSBAC was also used to clone and heterologously overexpress the tautomycetin BGC (Nah et al., 2015). In 2017, the PKS I pikromycin (60 kb) from *S. venezuelae* ATCC 15439 (Pyeon et al., 2017).

Successful cloning and heterologous expression of a variety of PKS and NRPS BGCs was also reported for the phage P1-derived Artificial Chromosome (PAC) derivative pESAC13 (Sosio et al., 2000). In 2013, the Bibb group used a PAC library to clone and express the entire 83.5 kb FK506 (tacrolimus) gene cluster from *S. tsukubaensis* NRRL 18488 in four different *S. coelicolor* A3(2) hosts (Jones et al., 2013). Another example of pESAC vector use is the cloning and heterologous expression of azalomycin and kanchanamycin, isolated respectively from *Streptomyces malaysiensis* DSM4137 and *Streptomyces olivaceus* Tü4018 (Hong et al., 2016).

Several methods for cloning NP BGCs using *in vivo* and *in vitro* homologous recombination as a key principle have been described. The Red/ET system, consisting of coliphage I-derived protein pair, Redα/Redβ, or the related RecET system using the RecE and RecT proteins from the Rac prophage can be used for *in vivo* homologous recombination (Muyrers et al., 2000; Sharan et al., 2009). Recombination is thereby achieved between a linear and circular DNA molecule in *E. coli*, LCHR, or between two linear molecules, LLHR (Zhang et al., 1998). One of the first applications of the RecET-mediated LLHR method was used to clone BGCs from *Photorhabdus luminescens* and express them in *E. coli* (Fu et al., 2012; Yin et al., 2015).

Based on linear-plus-linear homologous recombination, the TAR in the yeast *S. cerevisiae* was described for the first time by Kouprina and Larionov (Kouprina et al., 1997; Larionov et al., 1996) for cloning selective genomic loci from human DNA. Today, this method is used in many laboratories for the direct cloning and DNA assembly of bacterial genes and BGCs (Feng et al., 2011; Kim et al., 2010). Several successful applications of the TAR method to clone and heterologous by express BGCs from actinomycetes have been reported, for example: the NRPS BGC from the marine actinomycete *Saccharomonospora* sp. CNQ-490 encoding the dichlorinated lipopeptide antibiotic taromycin A (Yamanaka et al., 2014); the type II PKS pathway for the biosynthesis of enterocin from *Salinispora pacifica* (Bonet et al., 2015); the assembly of the grecocycline BGC from *Streptomyces* sp. Acta 1362 (Bilyk et al., 2016); and the aromatic PK antitumor agent cosmomycin (Larson et al., 2017). Among the *in vitro* techniques available for BGC cloning a variation of the TAR system is the CATCH method (Jiang et al., 2015). The

method is based on the Cas9 sequence-specific cleavage activity of bacterial chromosomes embedded in agarose plugs and on the subsequent ligation through Gibson assembly. The CATCH method was applied to clone the 36 kb jadomycin BGC from *S. venezuelae* and the 32 kb chlortetracycline BGC from *Streptomyces aureofaciens* (Jiang et al., 2015). Another promising example of an *in vitro* cloning method is represented by CISMR, which enables targeted isolation of contiguous megabase-sized segments of the genome by *in vitro* CRISPR digestion and is isolated via pulsed-field gel electrophoresis (PFGE) (Bennett-Baker and Mueller, 2017; Jiang et al., 2015; Liu et al., 2015).

Despite the strong impact on genome mining from the development of LLHR and TAR techniques on NP discovery, the presence of repetitive DNA sequences in NP BGCs can negatively affect the desired homologous recombination event. LLHR, TAR and CATCH also require highly specialized capturing vectors containing homology arms to the target BGC and a relatively large amount of genomic DNA is required for efficient recombination. A new strategy called DiPaC was developed for fast cloning and BGC refactoring. This method, relying on long-amplicon PCR with homology arms at the 5′-end and *in vitro* DNA assembly, was used to discover a new phenazine from *Serratia fonticola* and to transfer the native erythromycin BGC from *S. erythraea* into *Streptomyces* (Greunke et al., 2018).

3.2. Actinomycetes engineering to construct chassis strains for NP overproduction and discovery

Heterologous expression of BGCs has become an important approach for the discovery of NPs from silent gene clusters and for biosynthetic pathway refactoring (Gomez-Escribano and Bibb, 2011; Ren et al., 2017). This strategy relies on the use of a suitable host that is amenable to genetic manipulation, ideally fast growing, and with a low background of endogenously produced compounds (Baltz, 2010; Komatsu et al., 2010). Various actinomycetes, in particular *Streptomyces* species, have been engineered as chassis strains for the heterologous expression of NP BGCs (Gomez-Escribano and Bibb, 2014; Komatsu et al., 2013). In fact, due to the availability of a rich pool of precursors/substrates and toolkits for genetic manipulation, along with relatively easy cultivation, *Streptomyces* strains are suitable hosts for the production of NP derived from both other actinomycetes and plants (Nepal and Wang, 2018). Among *Streptomyces* species, *S. coelicolor* A(3)2 (Gomez-Escribano and Bibb, 2011; Zhou et al., 2012), *S. lividans* (Anné and Van Mellaert, 1993), *S. avermitilis* (Komatsu et al., 2010), *S. albus* (Fernández et al., 1996), and *S. roseosporus* (McHenney and Baltz, 1996) have been engineered by deletion of endogenous gene clusters, mutation of RNA polymerase, ribosomal proteins, and global regulators to optimize the expression of different specialized metabolites (Baltz, 2010; Liu et al., 2018).

The model organism *S. coelicolor* A(3)2 and its derivatives were used as the heterologous host to express and produce different classes of NPs as in the case of the BGCs encoding novobiocin and clorobiocin (Eustáquio et al., 2005), salinomycin (Yin et al., 2015), and R1 and roseophilin (Kimata et al., 2017), these last two being unique prodigiosin-related compounds.

Due to the small genome size (6.8 Mb), fast growth, and efficient transformation methods, *S. albus* J1074 has been widely used as a host for heterologous expression of numerous BGCs (Bilyk et al., 2016; Gullón et al., 2006; Winter et al., 2007). To improve *S. albus* properties as a surrogate host, a cluster free strain was developed (Myronovskyi et al., 2018). Fifteen dispensable BGCs were deleted and additional ΦC31 *attB* sites were introduced. The properties of this strain as a chassis for heterologous expression were demonstrated by the expression of several cryptic BGCs encoding NPs with a wide variety of biosynthetic precursors. The results obtained strongly indicate that this new strain, with a reduced secondary metabolite background and multiple insertion sites, can greatly facilitate the discovery of NPs through heterologous expression.

The availability of a panel of host organisms is crucial to test BGC expression. Studies conducted on the marine actinomycete *Salinispora* highlighted the need of a heterologous host different from *Streptomyces*. With this aim, Tang and co-worker engineered *S. tropica* CNB-440 to develop the first marine actinomycete heterologous host to complement *Streptomyces* hosts. The production of salinosporamides was abolished by replacing three biosynthetic genes (*sal*A-C) with a cassette containing the *S. coelicolor attB* site.

3.3. Biosensors

Among synthetic biology tools, biosensors have been increasingly used in metabolic engineering to implement biosynthetic pathways and for mining new NPs. One of the bottlenecks in metabolic engineering is the downstream analysis for monitoring the concentrations of a target product or metabolic intermediates during the strain development process (Guo et al., 2015; Yang et al., 2018; Yang et al., 2017; Zhang et al., 2015). Biosensors responsive to intracellular metabolites enable the detection of low abundance or unstable metabolites by transducing their concentration into an easily measurable signal (Lin et al., 2017; Liu et al., 2015). Biosensors can be classified based on their mechanisms of sensing the target compound and their output signal. In light of previous studies conducted on TetR-like proteins as repressors of secondary metabolite gene cluster expression (Li et al., 2016; Ostash et al., 2010), this protein was used as a system to develop a new biosensor in *Streptomyces* (Sun et al., 2017). Due to the capacity of TetR to bind the cluster-specified compound, TetR-like repressors coupled with the reporter gene *bpsA* were used to directly detect the production of specific secondary metabolites and not just the expression of its biosynthetic genes. The authors demonstrated in *S. lividans* that metabolite-sensing repressor-based biosensors can be used to assess the functional expression of secondary metabolite clusters for which expression is controlled by TetR-like proteins or other regulator families.

3.4. Cell-free systems

In order to overcome problems associated with the complexity of living organisms and the negative impacts of bioactive molecule overproduction to the host, an emerging technology for NP production is constituted by *in vitro*, cell-free platforms (Li et al., 2018; Lu, 2017). In these systems, it is possible to engineer biological parts to directly control the transcription, translation, and metabolism without using living cells. Initial protocols for *in vitro* transcription-translation systems (TX-TL) using a whole-cell extract are emerging as adaptable tools to synthesize recombinant proteins and to direct assemble NPs from biosynthetic genes (Goering et al., 2017).

A promising source for production of enzymes from Actinobacteria secondary metabolism *in vitro* is represented by *S. venezuelae*. Freemont and co-workers developed a high-activity *S. venezuelae* TX-TL system utilizing the *kasOp** promoter. The authors, modifying and improving the general protocol for *Streptomyces* TX-TL, showed that it is possible to synthesize a high-yield range of proteins from secondary metabolism such as enzymes of the oxytetracycline pathway (Moore et al., 2017).

3.5. Metabolic and morphology engineering using sub-lethal concentrations of antibiotics

As aforementioned, NP biosynthesis in actinomyetes is triggered and controlled by a complex growth and nutrient dependent network of signal molecules. Several studies demonstrated that NP with antibiotic activity represent a biological weapon protecting the producer strain from other competitors during stress conditions such as nutrient starvation. However, many other studies highlighted the action of antibiotics as a signaling compound. In fact, the exposure to sub-inhibitory concentrations of antibiotics induces changes in the transcriptome profile in many different bacterial species, inducing metabolite and

pigment production, differentiation, or motility (Davies, 2006; Davies et al., 2006; Romero et al., 2011). Using drugs targeting RNA polymerase or ribosomes such as rifampin, streptomycin, gentamicin, and erythromycin, Ochi and co-worker were able to select resistant mutants overproducing antibiotics. This ribosome engineering approach led to the activation of silent BGCs in different *Streptomyces* and antibiotics overproduction (Shima et al., 1996; Wang et al., 2008). A potentiated action on stimulation of antibiotic production was observed by using ribosome-targeting antibiotics at sub-inhibitory concentrations. In fact, due to the pleiotropic effect, the addition of lincomycin at 1/10 of its MIC to *S. coelicolor* A3(2) culture increased the production of actinorhodin by inducing the expression of the pathway-specific regulatory gene a*ctII*-ORF4 (Imai et al., 2015). Comparative transcriptome and proteome analysis revealed that lincomycin induces a global change in expression profile and enhanced the expression of the gene involved in lincomycin resistance mechanisms such as efflux ABC transporter and the 23S rRNA methyltransferase (Ishizuka et al., 2018).

The link between mycelial morphology and antibiotic production has been examined during fermentation in different producer strains such as *S. hygroscopicus* and *S. noursei*. In these strains, the high production of rapamycin and nystatin was associated with the formation of dispersed mycelium or dense pellets, respectively (Fang et al., 2000; Jonsbu et al., 2002). This evidence was used to demonstrate that antimicrobials can also act as versatile tools for *Streptomyces* mycelial morphology engineering and antibiotic production. A study performed on *S. coelicolor* A3(2) demonstrated the diverse effect that sub-lethal antibiotic concentration can exert on mycelium morphology and consequent activation or repression of NP production (Wang et al., 2017). Ding and co-worker demonstrated that supplementation of thiostrepton and spectinomycin at different concentrations induced a change of *S. coelicolor* A3(2) morphology and secondary metabolite production. Molecular analysis revealed that both antibiotics affect the expression of the cellulose synthase-like protein (*cslA*) gene, for which expression was previously associated with regulation of *Streptomyces* growth and differentiation (van Dissel et al., 2014; Xu et al., 2008).

4. Reprogramming biosynthetic pathways using synthetic biology approaches

4.1. Engineering PKS assembly lines

The use of synthetic biology techniques to reprogram PKS and NRPS assembly lines is a powerful method, both for NP modification and for the production of novel and new-to-nature compounds. Biosynthetic pathways involving modular enzymes such as type I PKSs or NRPSs were (and still are) the focus of synthetic biology inspired engineering. The core PKS/NRPS enzymes act as molecular assembly lines in which a clearly defined set of enzymatic domains is responsible for selecting, linking and in some cases modifying simple precursors into a highly complex molecule.

Due to their modular architecture, the rational engineering of type I PKS has been used to introduce diversity into polyketides, thus expanding the chemistry of living systems (Barajas et al., 2017; Kumar et al., 2003; Marsden et al., 1998). Different studies on PKS engineering demonstrated that is possible to form a functional hybrid enzyme by replacing individual domains or modules with one from a different polyketide synthase. Acyltransferase domain (AT) engineering via site-directed mutagenesis, domain swapping and cross-complementation of inactivated *cis*-PKS by *trans*-ATs have been largely explored in attempts to direct the incorporation of alternative extender units into polyketides (Musiol-Kroll and Wohlleben, 2018). Pioneering studies revealing the potential of AT engineering were conducted on the 6-deoxyerythronolide synthase (DEBS), which is responsible for synthesizing the macrolactone erythromycin in *S. erythraea*. Leadlay and co-workers demonstrated that a chimeric PKS, in which the AT domain from DEBS1-TE (malonyl-CoA specific) is replaced with the AT domain from module 2 of the rapamycin synthase (methylmalonyl-CoA specific), was fully functional and able to produce triketide lactone with the predicted modification (Oliynyk et al., 1996). Although AT swapping, in certain cases, represents a successful strategy to produce regioselectively modified analogs, numerous studies have demonstrated that AT domain exchange often results in chimeric PKSs being completely inactive or with reduced activity compared to the native system (Lau et al., 1999; Ruan et al., 1997). To explain the partial or complete loss of function in engineered PKSs, biochemical and structural studies are focusing their attention on the role of linker sequences in modular PKS. Khosla and co-workers demonstrated the importance of conserved residues in the protein – protein interface through alanine scanning mutagenesis of the conserved residues of the linker in DEBS module 3 (Yuzawa et al., 2012). More recently, a comprehensive *in vitro* investigation on inter-domain linkers in AT domain elucidated the optimal domain boundary to replace the original AT domains, showing that optimized boundaries can preserve protein stability and enzyme activity (Yuzawa et al., 2017).

An attractive strategy for engineering the incorporation of alternative extender units is the use of a freestanding *trans*-AT domain to complement an inactivated *cis*-AT mutant. Khosla and co-workers demonstrated that the *trans*-AT domain DszsAT from the disorazole BGC can complement an AT null mutant of DEBS1 *in vitro* (Dunn et al., 2014). Several studies demonstrated that these stand-alone *trans*-AT enzymes can be used as an approach to bypass the intrinsic selectivity of the *cis*-AT domain to introduce non-natural polyketide precursors. An example of *trans*-AT potential is represented by the discrete AT KirCII from the kirromycin antibiotic pathway which is able to install chemical handles *in vivo*, allyl- and propargyl-side chains, which can themselves serve as anchors for “click” chemistry (Musiol-Kroll et al., 2017). An increased amount of polyketides or derivatives can be produced by expression of engineered PKSs in which one or more whole modules are replaced while maintaining protein integrity and interactions (Weissman, 2016). For example, novel erythromycin derivatives from the native producing strain *S. erythraea* were obtained by exchanging the loading module of DEBS with that from the avermectin (Ave) PKS of *S. avermitilis* (Marsden et al., 1998). Production of milbemycins, a macrolide related to avermectin, was achieved through engineering of the avermectin biosynthetic pathway in the avermectin high-producing *S. avermitilis* strain. Due to the high gene cluster similarity, AveA1 and AveA3 modules were replaced with milbemycin MilA1 and MilA3, respectively, thus showing that it is possible to engineer PKSs and obtain a large amount of the desired product in the industrial strain (Kim et al., 2017).

Very recently, Keasling and co-workers released a web-based toolkit, ClusterCAD, to streamline and simplify the design of a chimeric PKS capable of producing a small compound of interest. By comparing PKS modules based on amino acid sequence similarity, it is possible to select candidate modules as a starting point in PKS engineering (Eng et al., 2018).

4.2. Engineering NRPS and hybrid NRPS-PKS assembly lines

NRPSs are a family of megaenzymes synthesizing peptides with a wide range of biological activities. NRPSs are organized in modules, where each of them is constituted by adenylation (A), condensation (C), and thiolation (T) domains working together to incorporate a specific amino acid into the growing peptide (Reimer et al., 2018). The modular architecture of NRPS assembly lines and the autonomy of each domain within the module has inspired module/domain engineering in order to generate new NPs (Süssmuth and Mainz, 2017). Despite numerous attempts, only a few examples of successful NRPS engineering can be counted (Brown et al., 2018; Williams, 2013). Due to its role in substrate recognition and binding, most of the initial NRPS engineering works were focused on the A-domain. Extensive A-domain reprogramming studies using directed evolution strategies have been conducted in

other Gram-positive bacteria such as *Bacillus* species (Evans et al., 2011; Zhang et al., 2013). However, in most of these cases the effects of mutations within the binding pocket substantially reduced the A-domain catalytic activity compared to the wild type. The reprogramming of hormaomycin A-domains was achieved *in vitro* by Piel's group through subdomain swapping. The altered substrate specificity was investigated by bioinformatics analysis which suggested a series of recombination events during evolution. Thus, A domains with altered substrate specificity were generated based on the putative recombination points (Crüsemann et al., 2013).

With the aim of establishing general guidelines to generate new artificial NRPSs, a strategy has been proposed for modifying NRPSs using defined exchange units (XUs) as functional units instead of modules (Bozhüyük et al., 2018). Bozhüyük *et al.* have shown that, by keeping a specific sequence motif in the linker areas it is possible to modify or generate new NRPS assembly lines. Despite *in vitro* evidence showing that it is possible to engineer NRPSs through domain or subdomain exchange, there have been no reports of successfully modified peptide products generated *in vivo* via these strategies.

In the NRPS–PKS complexes, the KS domain receives the aminoacyl chain from the CP domain of the upstream NRPS module, which will be condensed with the PKS extender substrate to generate an aminoacyl–acyl hybrid chain. When a PKS module is located upstream of an NRPS the acyl group from the PKS is condensed with the aminoacyl substrate by the C domain of the NRPS module. Thus, protein-protein interactions are necessary to achieve the correct functional hybridization within these hybrid assembly lines (Miyanaga et al., 2018). Recently, Abe and co-workers demonstrated that the strategy proposed by Bode and co-workers (Bozhüyük et al., 2018) to manipulate NRPSs and the general strategies to engineer PKSs can be also applied to hybrid NRPS-PKS engineering (Williams, 2013). Through sequence comparisons of three antimycin-type NRPS-PKSs, the authors rationally modified the antimycin assembly line module organization, obtaining depsipeptides with different lactone ring sizes and a yield 5-10 times higher than those reported in module assembly line engineering (Awakawa et al., 2018).

The potential interchangeability of catalytic domains and modules make these megaenzymes an attractive subject for combinatorial biosynthesis of novel bioactive compounds. However, despite being carried out for more than 20 years now, this approach has not yet left its infancy stage. Considering the world-wide efforts, the number of success stories is still relatively low and many unsuccessful attempts are never published. However, the dramatic increase of novel NRPS and PKS sequences originating from NP screening programs and untargeted whole genome sequencing, and the progress in understanding the structural blueprint of these megaenzymes and the interplay of the individual enzymatic domains (Keatinge-Clay, 2012; Kopp and Marahiel, 2007; Marahiel, 2016; Weissman, 2015) likely will provide novel approaches in the near future.

5. Concluding remarks and future perspectives

Over the past two decades, breakthroughs in whole genome sequencing revealed the untapped biosynthetic potential of actinomycetes, thus renewing the interest in these gifted bacteria and in NP discovery. Despite an increasing number of new NPs that have been discovered in past decades, the entire workflow from discovery to commercialization of NPs is challenging, time-consuming, and costly (Genilloud, 2017).

Systematic analyses involving multi-omics data and genome-scale metabolic network models have greatly contributed to exploring actinomycetes physiology, predicting targets for metabolic engineering, and re-designing metabolic flux to maximize the production yield of a target NP. The development of new molecular tools such as DNA assembly, recombineering, and genome editing technologies have reduced the time required and increased the efficiency of cloning and engineering BGCs. But there is still a huge performance gap between the tools available for model organisms and actinomycetes. The advent of new technologies has also facilitated the engineering of heterologous hosts, thus enabling NP production in genetically amenable strains. However, a major challenge is that many of the cloned BGCs are not expressed at all or only poorly expressed in the surrogate hosts. A prerequisite to overcome this challenge will be the characterization of regulatory elements controlling both transcription and translation; this may allow for the possibility to refactor BGCs and construct synthetic regulatory circuits for the fine and dynamic control of gene expression. Furthermore, novel-to-nature natural compounds can be produced by reprogramming PKS and NRPS assembly lines. In fact, the modular reconstruction of natural or modified PKSs and NRPSs could represent a powerful method, both for NP modification and to produce novel molecules. The integration of these technologies will help us in understanding of the complex gene regulatory networks and metabolic networks, thus allowing new NP discovery and production through BGCs engineering, pathway engineering, metabolic flux optimization, enzyme engineering, regulatory circuit rewiring, and host modification. Based on the advances in these topics, we expect to see increasing research on the discovery, development and more efficient production of new NPs. With our increased knowledge and information, we also expect to see generation of a "Google Map" for NPs similar to the "bio-based chemicals map" recently published (Lee et al., 2019).

In conclusion, advances in metabolic engineering and synthetic biology tools will provide a promising solution for the discovery and production of a variety of valuable active compounds and hopefully open a new golden age of NP discovery.

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Conflicts of interest

Y.T., T.W., and S.Y.L. are co-inventors on a patent application on an actinomycete CRIRSPR toolkit (WO2016150855) filed by the Technical University of Denmark.

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