

MINIREVIEW – Biotechnology & Synthetic Biology

Synthetic biology approaches to actinomycete strain improvement

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One sentence summary: Recent advances in synthetic biology driven engineering of the actinomycetes for the production of valuable drug compounds.

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ABSTRACT

Their biochemical versatility and biotechnological importance make actinomycete bacteria attractive targets for ambitious genetic engineering using the toolkit of synthetic biology. But their complex biology also poses unique challenges. This mini review discusses some of the recent advances in synthetic biology approaches from an actinomycete perspective and presents examples of their application to the rational improvement of industrially relevant strains.

Keywords: actinomycete; synthetic biology; actinobacteria; biosynthesis; biotechnology

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INTRODUCTION

Actinomycetes are chemically extremely talented bacteria, known for the exceptional diversity of their secondary metabolites (Barka et al. 2016; Jakubiec-Krzesniak et al. 2018). They are a major source for bioactive natural products, serving as starting points for clinically important drugs, in particular antibiotics (Genilloud 2017). Genome analyses show that individual strains of actinobacterial genomes typically contain the biosynthetic machinery for several dozen secondary metabolites, encoded in biosynthetic gene clusters in the genome ready for horizontal gene transfer as a compact functional unit (Lee et al. 2020). The emergence of advanced genome engineering techniques and the concepts and methodology of synthetic biology have stimulated renewed interest in exploiting the biosynthetic capabilities of actinomycetes, modifying and improving their performance as biotechnological sources of valuable drugs and drug precursors (Lee et al. 2019; Palazzotto et al. 2019; Zhao, Wang and Luo 2019). In this mini review, we highlight selected areas of synthetic biology as applied to actinomycete strain improvement. We focus on some areas of recent advances, as well as specific challenges encountered when engineering this specific group of microbes and offer an industrial perspective on the potential applications of synthetic biology to enhance the potential of actinobacteria as drug production hosts.

THE CHALLENGES OF HETEROLOGOUS EXPRESSION IN ACTINOMYCETES

Novel actinomycete strains, producing new compounds of interest with the potential of becoming industrial products, such as pharmaceuticals, crop-protecting agents or food additives are identified regularly (Onaka 2017; Kim et al. 2021). Initially, these novel strains of interest are not immediately used at an industrial scale, as their use presents several challenges due to a limited understanding of the strain and/or the product. The strain could have been identified after bioprospecting (Genilloud 2019; Nogawa, Lopez and Osada 2019) or after expression of a suitable DNA library (Zhang, Tang and Moore 2019). To translate the initial discovery into an industrial product, we need to study the producing strain and the biosynthetic pathway in considerable detail. The methods of synthetic biology are essential for this process. In particular, it is often necessary to genetically engineer the pathway or the host system by the introduction of heterologous genetic material.

Great progress has been made over the past decades in developing gene transfer systems for many actinomycete species and genera (Musiol-Kroll et al. 2019; Mitousis, Thoma and Musiol-Kroll 2020), and in devising effective genome editing tools (Alberti and Corre 2019). However, any actinomycete strain newly identified after bioprospecting is, by definition, poorly characterized and consequently often difficult to manipulate genetically. One can look at taxonomically related strains for inspiration; however, as a rule of thumb, developing a gene transfer system for a new strain is an unpredictable endeavor: successful introduction of heterologous DNA can either occur after a few months' effort, or a whole year can go by without progress. Even after a gene transfer system has been developed for a model strain, the genetic tools available in the actinomycete community will need to be tested and validated in the new strain.

A conceptually attractive alternative to developing a gene transfer system is to rely on production of the desired product in a robust and easy to manipulate host, after heterologous

expression of the necessary genes (Xu and Wright 2019; Kang and Kim 2021). This is a relatively straightforward approach that can benefit from a large selection of tools for introducing small and large segments in the host of choice (Tocchetti, Donadio and Sosio 2018), and successful heterologous expression can be confirmed relatively quickly. However, we still know very little about what controls formation of a heterologous product, from supply of precursors to enzymatic cofactors and from regulatory networks to efficient export of the final product. Consequently, heterologous expression of the native biosynthetic gene cluster (BGC) usually results in product titres lower than those obtained in the original host. These challenges necessitate a concerted approach at genetic engineering to optimize both the host production strain and the biosynthetic pathway itself for function in its new context.

PREDICTIVE MODELLING: METABOLISM AND BEYOND

Predictive metabolic modelling is a key ingredient in the rational engineering of improved production strains (Simeonidis and Price 2015). Such models can help identify competing metabolic pathways, optimal medium design and strategies for selecting high-producing mutants. Stoichiometric constraint-based models of metabolism have been particularly popular and successful for this purpose (Kim, Kim and Lee 2017; Gu et al. 2019), and multiple generations of increasingly refined and carefully curated models have been constructed for several important actinomycetes, including the model organisms *Streptomyces coelicolor* (Amara, Takano and Breiting 2018; Kumelj et al. 2019), *S. lividans* (D'Huys et al. 2012; Valverde, Gullón and Mellado 2018), and the industrial producer *S. clavuligerus* (Medema et al. 2010; Toro et al. 2018). In each of these, the models have been used not only to understand the basic biology of metabolism, but also to identify targets for strain engineering (Kim, Rocha and Maia 2018).

The availability of well-curated reference models for different *Streptomyces* species has meant that this group could be employed as a benchmark for automated model reconstruction methods (Wang et al. 2018). Automated model reconstruction has rapidly expanded the range of actinomycetes for which genome-scale models are available. A large-scale comparative study of genome-scale metabolic models of 37 species of actinobacteria identified conserved core networks of metabolism (Alam et al. 2011). Comparative modelling simultaneously optimizing compound biosynthesis and biomass production has been employed to highlight the differences in metabolic capacity for selected classes of secondary metabolites in different potential actinomycete hosts (Zakrzewski et al. 2012). More recently, stoichiometric metabolic models have been complemented with enzyme level constraints to obtain more precise predictions of engineering strategies for enhanced secondary metabolite production in *S. coelicolor* (Sulheim et al. 2020).

Metabolic models are not only useful as stand-alone predictive tools, but are particularly powerful when combined with the results of molecular profiling experiments, such as transcriptome or metabolome profiling (Blazier and Papin 2012; Kleessen et al. 2015; Graudenzi et al. 2018). The MORF tool (Springthorpe et al. 2020) can facilitate this integration by providing a single platform for interrogating multi-omics data, e.g. transcriptomics and proteomics, and then using these data as additional constraints for a linked genome-scale metabolic model.

Stoichiometric models of metabolism can be constructed from genome sequences, with very limited requirements for

experimental data. This allows their application even to organisms for which data availability limits more data-demanding modelling approaches. However, ensemble modelling strategies, which allow the construction of fully parameterized kinetic models of biological systems in the absence of extensive experimental data (Tsigkinopoulou, Baker and Breitling 2017; Tsigkinopoulou et al. 2018), are now making it possible to model additional aspects of actinomycete biology. For example, ensemble modeling has been employed for a detailed analysis of the γ -butyrolactone network in *S. coelicolor* (Tsigkinopoulou, Takano and Breitling 2020), a system with exciting potential as an engineered regulatory circuit to control secondary metabolic pathways (Biarnes-Carrera et al. 2018; Biarnes-Carrera, Breitling and Takano 2018).

REGULATORY ENGINEERING: SRNA AS AN EMERGING TOOL

Successful engineering of secondary metabolite production requires regulatory tools (Xia et al. 2020; Zhou, Ning and Luo 2020), which so far have not been extensively studied in actinomycetes, compared to traditional model organisms. For example, in *Streptomyces* or other antibiotic-producing actinomycetes only a few sRNA studies have been related to secondary metabolism. However, there has been much recent progress, making new regulatory tools accessible to actinomycete synthetic biologists. In *S. coelicolor*, an antisense RNA, Cnc2198.1, was found in the 5' region of the glutamine synthetase I gene *glnA*. The controlled overexpression of this antisense RNA resulted in a decrease in growth, protein synthesis and undecylprodigiosin production (D'Alia et al. 2010). Another sRNA, Scr4677, is encoded in the intergenic antisense strand between two co-transcribed regulatory genes (SCO4676 and SCO4677). Whilst disruption of each regulatory gene affected actinorhodin production, modulating Scr4677 levels showed no reproducible phenotypic differences (Hindra et al. 2014). Mikulík et al. (2014) found that deletion of *ssrS* gene in *S. coelicolor*, encoding the conserved 6S RNA which binds the β and β' subunits of RNA polymerase, reduced both the growth rate and actinorhodin production, which was accompanied by a lower expression of the pathway-specific *actII-ORF4* transcriptional regulator (Mikulík et al. 2014). Using RNA-seq, Liu et al. (2013) identified four sRNAs that were overexpressed in an industrial erythromycin-producing strain of *Saccharopolyspora erythraea* compared to the wild type strain (Liu et al. 2013). These genes are encoded on the complementary DNA strands of annotated ORFs, indicating a regulatory effect on iron transport (*sernc293*), terpene metabolism (*sernc350*), geosmin synthesis (*sernc351*) and polyketide biosynthesis (*sernc389*).

These fundamental advances are very promising for future applications, but few studies of sRNA-based regulatory engineering have been published in bacteria producing secondary metabolites of industrial interest (Chaudhary, Na and Lee 2015). In *Streptomyces*, the paired termini antisense (PTas)RNA strategy has been successfully applied to downregulate an actinorhodin biosynthetic gene in *S. coelicolor* (Uguru et al. 2013), to enhance antibiotic production through the inhibition of the transcriptional repressor DoxR in *Streptomyces peucetius* (Chaudhary et al. 2015a) and to elucidate the regulatory role of the cyclic AMP receptor protein (Crp) in *Streptomyces cinnamonensis* by comparing the monensin yields and transcriptomic profiles between control, *crp*-overexpressing and *crp*-silencing strains (Lin et al. 2020). There is a pressing need for more extensive studies to

identify sRNAs and elucidate their function in *Streptomyces* and other actinomycetes with a rich secondary metabolism.

CLONING, REFACTORIZING AND ASSEMBLY OF COMPLEX METABOLIC PATHWAYS

The biosynthetic capabilities of actinomycetes are encoded in large and complex BGCs. Successful strain engineering depends on our ability to manipulate these clusters efficiently, and a wide array of *in vivo* and *in vitro* methods have been established for this purpose. *In vivo* DNA assembly methods are based on the natural homologous recombination capacity in *Saccharomyces cerevisiae* or the Red/ET homologous recombination system. Transformation-associated recombination (TAR) cloning in yeast has been successfully employed to capture and heterologously express a range of natural product BGCs, including those of taromycin A, enterocin, greocycline and cosmomycin (Yamanaka et al. 2014; Bonet et al. 2015; Bilyk et al. 2016; Larson, Crüsemann and Moore 2017). In a study by Eyles, Vior and Truman (2018), an integrated approach of TAR and yeast-mediated engineering was used to refactor the bottromycin BGC from *Streptomyces scabies*. After capturing the *btm* cluster in a yeast/*Escherichia coli* shuttle vector using TAR, yeast-mediated homologous recombination was employed to assemble a variety of pathway combinations using PCR products, single stranded oligonucleotides and individual pathway fragments generated by restriction digestion. This approach allowed for the simultaneous introduction of promoters, gene deletions, targeted mutations and rearrangement of gene order, yielding a refactored BGC with a 120-fold increase in bottromycin-related metabolites in the heterologous host *S. coelicolor* (Eyles, Vior and Truman 2018).

Recently, a TAR-based and CRISPR/Cas9-mediated promoter engineering platform, termed multiplex *in vitro* Cas9-TAR (miCASTAR), has been developed by Kim, Rocha and Maia (2018) for the activation of the silent atolypenes BGC from *Amycolatopsis tolypomycina*. Following this approach, the *ato* cluster was digested *in vitro* by Cas9, reassembled in yeast using promoter cassettes with homology arms matching the specific Cas9 digestion sites and introduced into *Streptomyces albus* (Kim, Rocha and Maia 2018). Similar to TAR in yeast, the Red/ET homologous recombination system can be employed for assembly of complex BGCs in *E. coli*. Using Red/ET recombination, a variety of semi-synthetic promoters was incorporated into the bottromycin BGC, increasing the production titre by up to 50-fold (Horbál et al. 2018).

Examples of successful *in vitro* assembly methods include Gibson assembly, site-specific recombination-based tandem assembly (SSRTA) and methylation-assisted tailorable ends rational (MASTER) ligation (Zhang, Zhao and Ding 2011; Shao et al. 2013; Linares-Otoya et al. 2017). Gibson assembly is often coupled to *in vitro* applications of gene editing techniques for synthetic biology driven investigations. For example, a 36-kb jadomycin producing gene cluster from *Streptomyces venezuelae* was successfully cloned into an *E. coli* vector via Cas9-assisted targeting of chromosome segments (CATCH). RNA-guided Cas9 nuclease cleaves the target chromosome at two designated sites, prior to insertion of the target genome segment into a cloning vector by Gibson assembly. However, heterologous production of jadomycin was not reported (Jiang et al. 2015). In a similar manner, Li et al. (2017) effectively combined the CRISPR/Cas9 system with Gibson assembly to incorporate the pristinaamycin II (PII) BGC into *Streptomyces pristinaespiralis*; the resulting strain produced the highest titres of PII yet reported.

Direct pathway cloning (DiPaC), a combination of long-amplicon PCR and *in vitro* DNA recombination, successfully transplanted the native erythromycin BGC into a *Streptomyces* host for heterologous expression, with production confirmed via HPLC-MS (Greunke *et al.* 2018).

In addition, *in vitro* assembly methods can also be applied in the refactoring of biosynthetic pathways for the optimized production of valuable natural products in actinomycetes. Song *et al.* (2019) utilized the ExoCET (Exonuclease combined with RecET recombination) method to construct a plasmid containing a 79 kb artificial, GC-rich cluster for the heterologous production of insecticide spinosad within *S. albus* J1074.

Often, molecular engineering tools developed in other model organisms do not immediately work when applied in actinobacteria. Ye *et al.* (2020) present an example, showing how the fine-tuning of Cas9 expression is essential to obtain good performance of a CRISPR/Cas9 system in several *Streptomyces* species.

MANIPULATION OF PRODUCT EFFLUX FOR INCREASING YIELDS

While synthetic biology approaches have been used successfully in the engineering of metabolic pathways, resulting in bacteria that can produce high titres or novel products, the final step in the biosynthesis pathway, namely the export of the product into extracellular medium, is often overlooked, despite product efflux being a highly effective step to manipulate to increase yields in whole-cell catalysts (Kell *et al.* 2015; Lv *et al.* 2016; Soares-Silva *et al.* 2020). Most natural product BGCs in actinomycetes contain transporter genes of one type or another and their functions are almost exclusively involved in efflux, either in the secretion of the newly synthesized compound itself or in subsequent self-protection of the producing cell (Martín, Casqueiro and Liras 2005). Compared to the enzymatic machinery involved in the biosynthesis of natural products, the efflux proteins are rarely studied and only few examples of characterized systems are available, which have recently been reviewed in detail (Severi and Thomas 2019).

The simplest approach to empirically improve efflux/export is to overexpress the gene for the transporter. The streptogramin family of antibiotics provide the first example of this method, with pristinamycin produced by *Streptomyces pristinaespiralis*. The overexpression of a known resistance gene, *ptr*, that encodes an efflux pump of the major facilitator superfamily (MFS) resulted in 6 to 8-fold increase in yield of the antibiotic (Jin, Jin and Jin 2010). In a second example the production of the macrocyclic polyketide avermectin was also improved by about 50% by overexpression of the BGC-encoded ATP-binding cassette (ABC) transporter genes *avtAB* (Qiu *et al.* 2011). A third example comes from the production of oxytetracycline in *Streptomyces rimosus*, where the wild-type strain was modified to overexpress its three known self-resistance/export genes, of which two encode efflux proteins, and this resulted in oxytetracycline yields increasing over 150% (Yin *et al.* 2017).

Applying a synthetic biology approach to improve this process requires the ability to access or create variants of suitable transporter genes and apply an effective and simple high-throughput screen to assess function. An early approach of this kind was taken to try and build on the success of overexpressing *ptr* for increased pristinamycin production, where a DNA shuffling method was used to create a library of hybrid *ptr* genes taken from different high-producing strains of *S. pristinaespiralis*

(Jin *et al.* 2015). However, this strategy resulted in strains producing highly variable antibiotic yields with none of the hybrids surpassing the performance of the original *Ptr* protein, suggesting that larger libraries of random point mutants and/or *Ptr* homologues from different *Streptomyces* species, such as the *varS/snbR* gene from the virginiamycin biosynthetic cluster (Mast *et al.* 2011; Severi and Thomas 2019) would be more successful.

In an excellent example of the evolutionary approach, random mutagenesis has been used to evolve a transporter with an expanded substrate range for a biotechnological application (Bali, Genee and Sommer 2018). In this work, the function of a vitamin transporter unable to take up thiamine was linked to a growth-coupled biosensor that consisted of a thiamine-responsive riboswitch silencing a selectable marker. As the starting transporter cannot import thiamine, the riboswitch represses the marker and cells die on selective medium, thus providing a powerful genetic screen for gain-of-function mutants by growth on selective media. By screening a library of 60 000 variants made by error-prone PCR on this single transporter, the authors successfully found a transporter with the desired properties. Elegant screens of this nature could be used in the context of evolving antibiotic exporters with different affinities and specificities or just increased capacity for antibiotic export. Synthetic biology approaches can be used to create these candidate parts, and in this regard the *Streptomyces* genus provides an enormous reservoir of potential antibiotic exporter genes, with many genomes encoding around 1000 different transporters (around 10% of total coding capacity; Ren, Chen and Paulsen 2007; Zhou *et al.* 2016). It is highly likely that many of these function as either specific or multi-drug exporters to provide cross-resistance to natural products produced by other species in the complex soil niche, and synthetic biology methods now enable this enormous pool of untapped transporters to be sampled, characterized and exploited.

MOLECULAR PROFILING FOR ACTINOMYCETE SYNTHETIC BIOLOGY

Synthetic biology relies on the availability of molecular diagnostic tools for the analysis of the function of an engineered biological system. In the case of actinomycete synthetic biology, which largely focuses on the production of secondary metabolites, metabolomics is often the method of choice for this purpose. It can be applied in two major directions: either for the characterization of metabolic changes across the entire system, including primary metabolism, or to identify and quantify secondary metabolites and their precursors in a more targeted approach.

For example, a recent study on the impact of the expression of the actinorhodin BGC on the metabolome of *S. coelicolor* found pervasive effects across the metabolic network, including changes in precursors of secondary metabolites, as the result of the activity of a single biosynthetic pathway (Nitta *et al.* 2021). Quantitative analysis of the metabolome of *S. nodosus* allowed the rational engineering of improved amphotericin B production strains (Zhang *et al.* 2020).

Ideally, metabolomics is combined with other molecular profiling methods, e.g. measuring transcriptome dynamics and metabolic effects simultaneously. In actinomycete synthetic biology, such multi-omics analyses have a long tradition (Zhou *et al.* 2011; Lee *et al.* 2021), ranging from delimiting dispensable regions in *Streptomyces* genomes (Bu *et al.* 2020) and understanding the dynamic physiology of growth underlying the induction

of secondary metabolite production (Kim et al. 2020), to understanding the effect of extracellular cAMP as a regulator of secondary metabolite production (Nitta et al. 2020).

Engineered actinomycetes rarely are exclusive producers of the target chemical. More often, they co-produce relatively complex mixtures of secondary metabolites, creating additional analytical challenges for metabolomics profiling. Recent advances in LC-MS based approaches and tools for the fast identification of natural products, such as Global Natural Products Social molecular networking (GNPS) have revolutionized natural product research (Wang et al. 2016). Yet, the challenge remains of annotating the spectral information and identifying detected metabolites by linking isotopic patterns and fragmentation data to given molecular structures in secondary metabolites databases (Allard, Genta-Jouve and Wolfender 2017; Caesar et al. 2019). In the case of comprehensive metabolome profiles, Bayesian methods that take into account the plausible biochemical relationships among the ingredients of a sample can add further support to the annotation process—and provide a principled statistical assessment of the confidence in individual identifications (Tripathi et al. 2021).

Despite considerable advances, the structural elucidation of co-produced natural products remains a complex process leading in some cases to the assignment of incorrect structures. Computer Assisted Structural Elucidation (CASE) approaches, both commercial and academic, have been developed as powerful tools to help in the process through the generation of prioritized structural proposals, although these applications provide only planar (2D) structures and further improvements are still required to determine 3D structures (Burns, Mazzola and Reynolds 2019). Moreover, the DP4-AI software has emerged as a tool complementary to CASE for completely automated 3D structural elucidation starting from raw ^{13}C and ^1H NMR data (Howarth, Ermanis and Goodman 2020). Apart from these tools, databases including calculated/real NMR data and NMR structural features easily recognizable in NMR spectra have been developed to speed up the structural elucidation of new compounds and were reviewed recently (Pérez-Victoria, Martín and Reyes 2016).

Since then, other approaches based on the use of NMR in combination with structural features obtained from fast NMR experiments (Zani and Carroll 2017) or 2D heteronuclear single quantum coherence (HSQC) NMR spectra in combination with MS or ^1H NMR data have been developed. Perhaps the most remarkable effort in this sense has been the Small Molecule Accurate Recognition Technology (SMART) initiative (Reher et al. 2020). Using the HSQC spectrum of a sample as a query, this Artificial Intelligence-based tool allows the generation of structural hypotheses by comparative analysis with a library of > 100,000 real or computed spectra of natural products. The system also includes external links to other platforms including mass spectrometry, BGC and structural information in cases where known natural products are identified: GNPS, MiBIG (Kautsar et al. 2020) and the Natural Products Atlas (Van Santen et al. 2019). Lately, ^{13}C NMR-based tools have also been developed to dereplicate compounds in mixtures (MixONat; Bruguère et al. 2020) or the prediction of natural product classes (Martínez-Treviño et al. 2020).

APPLICATION CASE 1: CHALLENGES OF TETRACYCLINE PRODUCTION

The native tetracycline (TC) producing strains *Streptomyces aureofaciens* and *S. rimosus* are powerful industrial producers of

chlortetracycline (CTC) and oxytetracycline (OTC), respectively, aromatic polyketide metabolites of medical and agricultural importance (Chopra and Roberts 2001). Industrial strains for the production of these valuable compounds have been developed over many decades by intensive mutagenesis and strain selection approaches (Goodman 1985). The main challenge today is to further improve the yield of target TCs, particularly by increasing the rate of conversion of the carbon source, mostly from media containing starch, while reducing the presence of undesired impurities. OTC-producing *S. rimosus* strains are generally easily transformable, fast-growing strains compared to many other streptomycetes and numerous industrially useful molecular biology tools and microbiology methods are available, including the latest genome editing technologies, which make these strains attractive chassis for a synthetic biology approach (Jia et al. 2017; Carrillo Rincón et al. 2018).

Early successes were realized when a chlortetracycline BGC was heterologously expressed in the industrial strain *S. rimosus* 461, and additional engineering to achieve overexpression of a cluster-situated activator led to 38-fold increase in titre of CTC compared to the original producer (Wang et al. 2019). There is at present a critical need for novel antibiotics, to address the rapid spread of antibacterial resistance, and TC intermediates and shunt products may represent a very attractive source of drug leads (Martens and Demain 2017; Petković, Lukežič and Šuškić 2017). Because of the rapid development of synthetic biology approaches, it is now possible to generate novel TC scaffolds through rational approaches (Wang et al. 2012; Cummings, Breitling and Takano 2014; Lukežič et al. 2019). An important successful example is provided by the work of Wang et al. (2012) who used three structurally diverse natural tetracycline BGCs encoding antibiotic OTC, the antitumor SF2575 and dactylocycline as the starting point for engineering. A number of new tetracycline compounds were obtained from the engineered host-pathway pairs (Wang et al. 2012). Another example is the introduction of a novel starter unit into the backbone of the atypical tetracycline chelocardin produced by *Amycolatopsis sulphurea*, which results in a potent antibacterial compound currently in preclinical evaluation (Lešnik et al. 2015).

However, it is becoming clear that polyketide synthase (PKS) enzymatic complexes catalysing the formation of TCs are not very robust, i.e. even a small modification of the targeted enzyme(s) may inhibit the catalytic activity of these complex enzymes, consequently disabling the functionality of the entire PKS complex. Most often, this results in a significant drop in the titres of target intermediate(s) or even in a derailment of the whole Type II PKS complex. Moreover, we have not yet completely elucidated the preferred order of the enzymatic reactions, particularly those related to the late stages in TC biosynthesis (post-PKS biosynthesis), when functional groups are added to the basic polyketide backbone (Wang et al. 2013). To fully capitalize on the potential of synthetic biology for improved and diversified tetracycline production, there is a need for more in-depth understanding of the catalytic mechanisms of the enzyme complexes involved.

APPLICATION CASE 2: PRODUCING RIPPS

Ribosomally synthesized and posttranslationally modified peptides (RIPPs) are one of the major groups of bioactive compounds found in actinomycetes. Because of its well-studied genetic background and a plethora of available molecular tools for strain engineering, *S. coelicolor* is one of the most frequently used hosts for heterologous expression of RiPP BGCs. Thiostreptamide S4, which belongs to a class of structurally novel RiPPs

characterized by a contiguous sequence of thioamide groups, was originally isolated from *Streptomyces* sp. NRRL S-4 and heterologously produced in *S. coelicolor* M1146 at levels comparable to the native producer (Frattaruolo et al. 2017). Similarly, thiovarsolins, a second class of thioamidated RiPPs, were discovered in the genetically intractable *S. varsoviensis* and heterologously produced in *S. coelicolor* M1146 (Santos-Aberturas et al. 2019). In case of the leupeptin BGC from *S. leeuwenhoekii* C34T, constitutive expression of the BGC in the heterologous host *S. coelicolor* was necessary for sufficient quantities of leupeptin for enabling structural elucidation (Gomez-Escribano et al. 2019).

Aside from *S. coelicolor*, a variety of RiPP-encoding BGCs have been successfully expressed in other heterologous *Streptomyces* hosts in recent years. Using heterologous expression, it was possible to increase production of MS-271 18-fold and confirm that epimerization of the C-terminal L-tryptophan into its D-form involves a novel, uncharacterized epimerase (Feng et al. 2018). *S. lividans* TK24 has been also employed to produce a library of natural and designed derivatives of thioviridamide/neothioviridamide, an anti-cancer agent of RiPPs origin with polythioamide backbone. A total of eight of the designed molecules exhibit cytotoxicity twice that of the wild-type natural product (Kudo et al. 2019). Derivatization of the RiPP cinnamycin was achieved by incorporation of three noncanonical amino acid residues into two different positions of the core peptide. The methodology was developed using *S. albus* as a heterologous host via co-expression of the *cin* BGC and *pylRS/tRNA^{Pyl}* pair. In *S. albus*, the titre of deoxycinnamycin produced was 5-fold greater relative to the natural mixture of cinnamycins produced by the same heterologous host (Lopatniuk, Myronovskiy and Luzhetskyy 2017).

As the metabolic engineering of actinomycetes is frequently challenging, heterologous expression in *E. coli* is an important tool when probing the mechanisms of RiPP biosynthesis pathways derived from actinomycetes (Hwang et al. 2014). For example, a recent study investigating the installation of azole rings within the sulfomycin biosynthesis pathway of *S. viridochromogenes* employed *E. coli* as part of a heterologous expression strategy to examine the roles of a number of key residues within the core peptide of precursor peptide, SulA (Du et al. 2020). In addition, the characterization of a novel venezuelin-like lanthipeptide was enabled by the establishment of an *E. coli*-based heterologous production system. Consequently, the study provided greater insight into the mechanism of class IV lanthipeptide biosynthesis (Hegemann and Van Der Donk 2018). The discovery of a unique two-component lantibiotic, roseocin, was in part facilitated by the partial *in vitro* reconstitution of the cluster within *E. coli*. The resulting posttranslationally modified lanthipeptides (RosA1 and RosA2) obtained from heterologous expression within *E. coli* were subsequently converted into bioactive roseocin via *in vitro* cleavage using commercial proteases (Singh, Chaudhary and Sareen 2020).

CONCLUSION

The recent great technological advances in the area of synthetic biology of actinomycetes present a great promise for a revolutionary transformation of the biotechnological production of powerful new drugs. They can facilitate the biochemical characterization of new compounds, help enhance their production to economically viable levels, support the chemical diversification of initial chemical scaffolds, and accelerate the transfer of new compounds to industrial production. The examples discussed illustrate the range of the techniques and concepts

that contribute to this technological revolution, but they also highlight the numerous areas that still require further research before the full potential of synthetic biology can be realized. Actinomycetes, with their immense chemical diversity and their unique challenges and opportunities for biosynthetic engineering, will remain at the forefront of these developments.

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REFERENCES

- Alam MT, Medema MH, Takano E et al. Comparative genome-scale metabolic modeling of actinomycetes: the topology of essential core metabolism. *FEBS Lett* 2011;585:2389–94.
- Alberti F, Corre C. Editing streptomycete genomes in the CRISPR/Cas9 age. *Nat Prod Rep* 2019;36:1237–48.
- Allard PM, Genta-Jouve G, Wolfender JL. Deep metabolome annotation in natural products research: towards a virtuous cycle in metabolite identification. *Curr Opin Chem Biol* 2017;36:40–9.
- Amara A, Takano E, Breitling R. Development and validation of an updated computational model of *Streptomyces coelicolor* primary and secondary metabolism. *BMC Genomics* 2018;19:1–9.
- Bali AP, Genee HJ, Sommer MOA. Directed evolution of membrane transport using synthetic selections. *ACS Synthetic Biol* 2018;7:789–93.
- Barka EA, Vatsa P, Sanchez L et al. Taxonomy, physiology, and natural products of actinobacteria. *Microbiol Mol Biol Rev* 2016;80:1–43.
- Biarnes-Carrera M, Breitling R, Takano E. Detection and quantification of butyrolactones from streptomycetes. *Methods Mol Biol* 2018;1673:117–28.
- Biarnes-Carrera M, Lee CK, Nihira T et al. Orthogonal regulatory circuits for *Escherichia coli* based on the γ -butyrolactone system of *Streptomyces coelicolor*. *ACS Synthetic Biol* 2018;7:1043–55.
- Bilyk O, Sekurova ON, Zotchev SB et al. Cloning and heterologous expression of the grecoacycline biosynthetic gene cluster. Virolle M-J (ed.). *PLoS One* 2016;11:e0158682.

- Blazier AS, Papin JA. Integration of expression data in genome-scale metabolic network reconstructions. *Front Physiol* 2012;**3**:299.
- Bonet B, Teufel R, Crüsemann M et al. Direct capture and heterologous expression of salinispora natural product genes for the biosynthesis of enterocin. *J Nat Prod* 2015;**78**:539–42.
- Bruguère A, Derbré S, Dietsch J et al. MixONat, a software for the dereplication of mixtures based on ¹³C NMR spectroscopy. *Anal Chem* 2020;**92**:8793–801.
- Bu QT, Li YP, Xie H et al. Comprehensive dissection of dispensable genomic regions in *Streptomyces* based on comparative analysis approach. *Microb Cell Fact* 2020;**19**, DOI: 10.1186/s12934-020-01359-4.
- Burns DC, Mazzola EP, Reynolds WF. The role of computer-assisted structure elucidation (CASE) programs in the structure elucidation of complex natural products. *Nat Prod Rep* 2019;**36**:919–33.
- Caesar LK, Kellogg JJ, Kvalheim OM et al. Opportunities and limitations for untargeted mass spectrometry metabolomics to identify biologically active constituents in complex natural product mixtures. *J Nat Prod* 2019;**82**:469–84.
- Carrillo Rincón AF, Magdevska V, Kranjc L et al. Production of extracellular heterologous proteins in *Streptomyces rimosus*, producer of the antibiotic oxytetracycline. *Appl Microbiol Biotechnol* 2018;**102**:2607–20.
- Chaudhary AK, Na D, Lee EY. Rapid and high-throughput construction of microbial cell-factories with regulatory noncoding RNAs. *Biotechnol Adv* 2015;**33**:914–30.
- Chaudhary AK, Pokhrel AR, Hue NT et al. Paired-termini antisense RNA mediated inhibition of DoxR in *Streptomyces peucetius* ATCC 27952. *Biotechnol Bioprocess Eng* 2015a;**20**:381–8.
- Chopra I, Roberts M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 2001;**65**:232–60.
- Cummings M, Breitling R, Takano E. Steps towards the synthetic biology of polyketide biosynthesis. *FEMS Microbiol Lett* 2014;**351**:116–25.
- D'Alia D, Nieselt K, Steigele S et al. Noncoding RNA of glutamine synthetase I modulates antibiotic production in *Streptomyces coelicolor* A3(2). *J Bacteriol* 2010;**192**:1160–4.
- D'Huys PJ, Lule I, Vercammen D et al. Genome-scale metabolic flux analysis of *Streptomyces lividans* growing on a complex medium. *J Biotechnol* 2012;**161**:1–13.
- Du Y, Qiu Y, Meng X et al. A heterotrimeric dehydrogenase complex functions with 2 distinct YcaO proteins to install 5 azole heterocycles into 35-membered sulfomycin thiopeptides. *J Am Chem Soc* 2020;**142**:8454–63.
- Eyles TH, Vior NM, Truman AW. Rapid and robust yeast-mediated pathway refactoring generates multiple new bottromycin-related metabolites. *ACS Synthetic Biol* 2018;**7**:1211–8.
- Feng Z, Ogasawara Y, Nomura S et al. Biosynthetic gene cluster of a d-tryptophan-containing lasso peptide, MS-271. *ChemBioChem* 2018;**19**:2045–8.
- Frattaruolo L, Lacrete R, Cappello AR et al. A genomics-based approach identifies a thioviridamide-like compound with selective anticancer activity. *ACS Chem Biol* 2017;**12**:2815–22.
- Genilloud O. Actinomycetes: still a source of novel antibiotics. *Nat Prod Rep* 2017;**34**:1203–32.
- Genilloud O. Natural products discovery and potential for new antibiotics. *Curr Opin Microbiol* 2019;**51**:81–7.
- Gomez-Escribano JP, Castro JF, Razmilic V et al. Heterologous expression of a cryptic gene cluster from *Streptomyces leeuwenhoekii* C34T yields a novel lasso peptide, leepeptin. *Appl Environ Microbiol* 2019;**85**, DOI: 10.1128/AEM.01752-19.
- Goodman JJ. *Fermentation and Mutational Development of the Tetracyclines*. Springer, Berlin, Heidelberg, 1985, 5–57.
- Graudenzi A, Maspero D, Di Filippo M et al. Integration of transcriptomic data and metabolic networks in cancer samples reveals highly significant prognostic power. *J Biomed Inform* 2018;**87**:37–49.
- Greunke C, Duell ER, D'Agostino PM et al. Direct Pathway Cloning (DiPaC) to unlock natural product biosynthetic potential. *Metab Eng* 2018;**47**:334–45.
- Gu C, Kim GB, Kim WJ et al. Current status and applications of genome-scale metabolic models. *Genome Biol* 2019;**20**:1–18.
- Hegemann JD, Van Der Donk WA. Investigation of substrate recognition and biosynthesis in class IV lanthipeptide systems. *J Am Chem Soc* 2018;**140**:5743–54.
- Hindra, Moody MJ, Jones SE et al. Complex intra-operonic dynamics mediated by a small RNA in *Streptomyces coelicolor*. Hoskisson P (ed.). *PLoS One* 2014;**9**:e85856.
- Horbal L, Marques F, Nadmid S et al. Secondary metabolites over-production through transcriptional gene cluster refactoring. *Metab Eng* 2018;**49**:299–315.
- Howarth A, Ermanis K, Goodman JM. DP4-AI automated NMR data analysis: straight from spectrometer to structure. *Chem Sci* 2020;**11**:4351–9.
- Hwang KS, Kim HU, Charusanti P et al. Systems biology and biotechnology of *Streptomyces* species for the production of secondary metabolites. *Biotechnol Adv* 2014;**32**:255–68.
- Jakubiec-Krzyszniak K, Rajnisz-Mateusiak A, Guspil A et al. Secondary metabolites of actinomycetes and their antibacterial, antifungal and antiviral properties. *Pol J Microbiol* 2018;**67**:259–72.
- Jia H, Zhang L, Wang T et al. Development of a crispr/cas9-mediated gene-editing tool in *streptomyces rimosus*. *Microbiol (United Kingdom)* 2017;**163**:1148–55.
- Jiang W, Zhao X, Gabrieli T et al. Cas9-assisted targeting of chromosome segments CATCH enables one-step targeted cloning of large gene clusters. *Nat Commun* 2015;**6**:1–8.
- Jin QC, Shen N, Yin H et al. DNA shuffling of ptr resistance gene leads to improved pristinamycin production in *Streptomyces pristinaespiralis*. *Mol Biol* 2015;**49**:253–9.
- Jin Z, Jin X, Jin Q. Conjugal transferring of resistance gene ptr for improvement of pristinamycin-producing *Streptomyces pristinaespiralis*. *Appl Biochem Biotechnol* 2010;**160**:1853–64.
- Kang HS, Kim ES. Recent advances in heterologous expression of natural product biosynthetic gene clusters in *Streptomyces* hosts. *Curr Opin Biotechnol* 2021;**69**:118–27.
- Kautsar SA, Blin K, Shaw S et al. MIBiG 2.0: a repository for biosynthetic gene clusters of known function. *Nucleic Acids Res* 2020;**48**:D454–8.
- Kell DB, Swainston N, Pir P et al. Membrane transporter engineering in industrial biotechnology and whole cell biocatalysis. *Trends Biotechnol* 2015;**33**:237–46.
- Kim JH, Lee N, Hwang S et al. Discovery of novel secondary metabolites encoded in actinomycete genomes through coculture. *J Ind Microbiol Biotechnol* 2021, DOI: 10.1093/jimb/kuua001.
- Kim OD, Rocha M, Maia P. A review of dynamic modeling approaches and their application in computational strain optimization for metabolic engineering. *Front Microbiol* 2018;**9**:1690.
- Kim W, Hwang S, Lee N et al. Transcriptome and translome profiles of *Streptomyces* species in different growth phases. *Sci Data* 2020;**7**, DOI: 10.1038/s41597-020-0476-9.

- Kim WJ, Kim HU, Lee SY. Current state and applications of microbial genome-scale metabolic models. *Curr Opin Syst Biol* 2017;**2**:10–8.
- Kleessen S, Irgang S, Klie S et al. Integration of transcriptomics and metabolomics data specifies the metabolic response of *Chlamydomonas* to rapamycin treatment. *Plant J* 2015;**81**:822–35.
- Kudo K, Koiwai H, Kagaya N et al. Comprehensive derivatization of thioviridamides by heterologous expression. *ACS Chem Biol* 2019;**14**:1135–40.
- Kumelj T, Sulheim S, Wentzel A et al. Predicting strain engineering strategies using iKS1317: a genome-scale metabolic model of *Streptomyces coelicolor*. *Biotechnol J* 2019;**14**:1800180.
- Larson CB, Crüsemann M, Moore BS. PCR-independent method of transformation-associated recombination reveals the cosmomycin biosynthetic gene cluster in an ocean streptomycete. *J Nat Prod* 2017;**80**:1200–4.
- Lee N, Hwang S, Kim J et al. Mini review: genome mining approaches for the identification of secondary metabolite biosynthetic gene clusters in *Streptomyces*. *Comput Struct Biotechnol J* 2020;**18**:1548–56.
- Lee N, Hwang S, Kim W et al. Systems and synthetic biology to elucidate secondary metabolite biosynthetic gene clusters encoded in *Streptomyces* genomes. *Nat Prod Rep* 2021, DOI: 10.1039/d0np00071j.
- Lee N, Hwang S, Lee Y et al. Synthetic biology tools for novel secondary metabolite discovery in streptomycetes. *J Microbiol Biotechnol* 2019;**29**:667–86.
- Lešnik U, Lukežič T, Podgoršek A et al. Construction of a new class of tetracycline lead structures with potent antibacterial activity through biosynthetic engineering. *Angew Chem* 2015;**127**:4009–12.
- Li L, Zheng G, Chen J et al. Multiplexed site-specific genome engineering for overproducing bioactive secondary metabolites in actinomycetes. *Metab Eng* 2017;**40**:80–92.
- Linares-Otoya L, Linares-Otoya V, Armas-Mantilla L et al. Identification and heterologous expression of the kocurin biosynthetic gene cluster. *Microbiol (United Kingdom)* 2017;**163**:1409–14.
- Lin C-Y, Zhang Y, Wu J-H et al. Regulatory patterns of Crp on monensin biosynthesis in *Streptomyces cinnamonensis*. *Microorganisms* 2020;**8**:271.
- Liu W-B, Shi Y, Yao L-L et al. Prediction and characterization of small non-coding RNAs related to secondary metabolites in *Saccharopolyspora erythraea*. Virolle M-J (ed.). *PLoS One* 2013;**8**:e80676.
- Lopatniuk M, Myronovskiy M, Luzhetskyy A. *Streptomyces albus*: a new cell factory for non-canonical amino acids incorporation into ribosomally synthesized natural products. *ACS Chem Biol* 2017;**12**:2362–70.
- Lukežič T, Fayad AA, Bader C et al. Engineering atypical tetracycline formation in *Amycolatopsis sulphurea* for the production of modified chelocardin antibiotics. *ACS Chem Biol* 2019;**14**:468–77.
- Lv H, Li J, Wu Y et al. Transporter and its engineering for secondary metabolites. *Appl Microbiol Biotechnol* 2016;**100**:6119–30.
- Martens E, Demain AL. The antibiotic resistance crisis, with a focus on the United States. *J Antibiot (Tokyo)* 2017;**70**:520–6.
- Martínez-Treviño SH, Uc-Cetina V, Fernández-Herrera MA et al. Prediction of natural product classes using machine learning and ¹³C NMR spectroscopic data. *J Chem Inf Model* 2020;**60**:3376–86.
- Martín JF, Casqueiro J, Liras P. Secretion systems for secondary metabolites: how producer cells send out messages of intercellular communication. *Curr Opin Microbiol* 2005;**8**:282–93.
- Mast Y, Weber T, Gözl M et al. Characterization of the ‘pristinamycin supercluster’ of *Streptomyces pristinaespiralis*. *Microb Biotechnol* 2011;**4**:192–206.
- Medema MH, Trefzer A, Kovalchuk A et al. The sequence of a 1.8-Mb bacterial linear plasmid reveals a rich evolutionary reservoir of secondary metabolic pathways. *Genome Biol Evol* 2010;**2**:212–24.
- Mikulík K, Bobek J, Zídková J et al. 6S RNA modulates growth and antibiotic production in *Streptomyces coelicolor*. *Appl Microbiol Biotechnol* 2014;**98**:7185–97.
- Mitousis L, Thoma Y, Musiol-Kroll EM. An update on molecular tools for genetic engineering of actinomycetes—the source of important antibiotics and other valuable compounds. *Antibiotics* 2020;**9**:1–31.
- Musiol-Kroll EM, Tocchetti A, Sosio M et al. Challenges and advances in genetic manipulation of filamentous actinomycetes—the remarkable producers of specialized metabolites. *Nat Prod Rep* 2019;**36**:1351–69.
- Nitta K, Breitling R, Takano E et al. Investigation of the effects of actinorhodin biosynthetic gene cluster expression and a rpoB point mutation on the metabolome of *Streptomyces coelicolor* M1146. *J Biosci Bioeng* 2021;**131**, DOI: 10.1016/j.jbiosc.2021.01.002.
- Nitta K, Carratore F Del, Breitling R et al. Multi-omics analysis of the effect of cAMP on actinorhodin production in *Streptomyces coelicolor*. *Front Bioeng Biotechnol* 2020;**8**, DOI: 10.3389/fbioe.2020.595552.
- Nogawa T, Lopez JAV, Osada H. *Microbial Fraction Library: A Screening Source for Drug Discovery. Cutting-Edge Organic Synthesis and Chemical Biology of Bioactive Molecules*. Springer; Singapore, 2019, 1–20.
- Onaka H. Novel antibiotic screening methods to awaken silent or cryptic secondary metabolic pathways in actinomycetes. *J Antibiot (Tokyo)* 2017;**70**:865–70.
- Palazzotto E, Tong Y, Lee SY et al. Synthetic biology and metabolic engineering of actinomycetes for natural product discovery. *Biotechnol Adv* 2019;**37**:107366.
- Pérez-Victoria I, Martín J, Reyes F. Combined LC/UV/MS and NMR strategies for the dereplication of marine natural products. *Planta Med* 2016;**82**:857–71.
- Petković H, Lukežič T, Šušković J. Biosynthesis of oxytetracycline by *Streptomyces rimosus*: past, present and future directions in the development of tetracycline antibiotics. *Food Technol Biotechnol* 2017;**55**:3–13.
- Qiu J, Zhuo Y, Zhu D et al. Overexpression of the ABC transporter AvtAB increases avermectin production in *Streptomyces avermitilis*. *Appl Microbiol Biotechnol* 2011;**92**:337–45.
- Reher R, Kim HW, Zhang C et al. A convolutional neural network-based approach for the rapid annotation of molecularly diverse natural products. *J Am Chem Soc* 2020;**142**:4114–20.
- Ren Q, Chen K, Paulsen IT. TransportDB: a comprehensive database resource for cytoplasmic membrane transport systems and outer membrane channels. *Nucleic Acids Res* 2007;**35**:D274–9.
- Santos-Aberturas J, Chandra G, Frattaruolo L et al. Uncovering the unexplored diversity of thioamidated ribosomal peptides in Actinobacteria using the RiPPER genome mining tool. *Nucleic Acids Res* 2019;**47**:4624–37.
- Severi E, Thomas GH. Antibiotic export: transporters involved in the final step of natural product production. *Microbiol (United Kingdom)* 2019;**165**:805–18.

- Shao Z, Rao G, Li C et al. Refactoring the silent spectinabilin gene cluster using a plug-and-play scaffold. *ACS Synth Biol* 2013;**2**:662–9.
- Simeonidis E, Price ND. Genome-scale modeling for metabolic engineering. *J Ind Microbiol Biotechnol* 2015;**42**:327–38.
- Singh M, Chaudhary S, Sareen D. Roseocin, a novel two-component lantibiotic from an actinomycete. *Mol Microbiol* 2020;**113**:326–37.
- Soares-Silva I, Ribas D, Sousa-Silva M et al. Membrane transporters in the bioproduction of organic acids: state of the art and future perspectives for industrial applications. *FEMS Microbiol Lett* 2020;**367**:118.
- Song C, Luan J, Cui Q et al. Enhanced heterologous spinosad production from a 79-kb synthetic multioperon assembly. *ACS Synth Biol* 2019;**8**:137–47.
- Springthorpe V, Leaman R, Sifouna D et al. MORF: an online tool for exploring microbial cell responses using multi-omics analysis. *Access Microbiol* 2020;**2**:763.
- Sulheim S, Kumelj T, van Dissel D et al. Enzyme-constrained models and omics analysis of *Streptomyces coelicolor* reveal metabolic changes that enhance heterologous production. *iScience* 2020;**23**:101525.
- Tocchetti A, Donadio S, Sosio M. Large inserts for big data: artificial chromosomes in the genomic era. *FEMS Microbiol Lett* 2018;**365**:64.
- Toro L, Pinilla L, Avignone-Rossa C et al. An enhanced genome-scale metabolic reconstruction of *Streptomyces clavuligerus* identifies novel strain improvement strategies. *Bioprocess Biosyst Eng* 2018;**41**:657–69.
- Tripathi A, Vázquez-Baeza Y, Gauglitz JM et al. Chemically informed analyses of metabolomics mass spectrometry data with Qemistree. *Nat Chem Biol* 2021;**17**:146–51.
- Tsigkinopoulou A, Baker SM, Breitling R. Respectful modeling: addressing uncertainty in dynamic system models for molecular biology. *Trends Biotechnol* 2017;**35**:518–29.
- Tsigkinopoulou A, Hawari A, Uttley M et al. Defining informative priors for ensemble modeling in systems biology. *Nat Protoc* 2018;**13**:2643–63.
- Tsigkinopoulou A, Takano E, Breitling R. Unravelling the γ -butyrolactone network in *Streptomyces coelicolor* by computational ensemble modelling. Csikász-Nagy A (ed.). *PLoS Comput Biol* 2020;**16**:e1008039.
- Uguru GC, Mondhe M, Goh S et al. Synthetic RNA silencing of actinorhodin biosynthesis in *Streptomyces coelicolor* A3(2). Isalan M (ed.). *PLoS One* 2013;**8**:e67509.
- Valverde JR, Gullón S, Mellado RP. Modelling the metabolism of protein secretion through the Tat route in *Streptomyces lividans*. *BMC Microbiol* 2018;**18**:1–11.
- Van Santen JA, Jacob G, Singh AL et al. The natural products atlas: an open access knowledge base for microbial natural products discovery. *ACS Central Sci* 2019;**5**:1824–33.
- Wang H, Marcišauskas S, Sánchez BJ et al. RAVEN 2.0: a versatile toolbox for metabolic network reconstruction and a case study on *Streptomyces coelicolor*. *PLoS Comput Biol* 2018;**14**:e1006541.
- Wang M, Carver JJ, Phelan VV et al. Sharing and community curation of mass spectrometry data with global natural products social molecular networking. *Nat Biotechnol* 2016;**34**:828–37.
- Wang P, Bashiri G, Gao X et al. Uncovering the enzymes that catalyze the final steps in oxytetracycline biosynthesis. *J Am Chem Soc* 2013;**135**:7138–41.
- Wang P, Kim W, Pickens LB et al. Heterologous expression and manipulation of three tetracycline biosynthetic pathways. *Angew Chem* 2012;**124**:11298–302.
- Wang X, Yin S, Bai J et al. Heterologous production of chlortetracycline in an industrial grade *Streptomyces rimosus* host. *Appl Microbiol Biotechnol* 2019;**103**:6645–55.
- Xia H, Li X, Li Z et al. The application of regulatory cascades in *Streptomyces*: yield enhancement and metabolite mining. *Front Microbiol* 2020;**11**, DOI: 10.3389/fmicb.2020.00406.
- Xu M, Wright GD. Heterologous expression-facilitated natural products' discovery in actinomycetes. *J Ind Microbiol Biotechnol* 2019;**46**:415–31.
- Yamanaka K, Reynolds KA, Kersten RD et al. Direct cloning and refactoring of a silent lipopeptide biosynthetic gene cluster yields the antibiotic taromycin A. *Proc Natl Acad Sci* 2014;**111**:1957–62.
- Ye S, Enghiad B, Zhao H et al. Fine-tuning the regulation of Cas9 expression levels for efficient CRISPR-Cas9 mediated recombination in *Streptomyces*. *J Ind Microbiol Biotechnol* 2020;**47**:413–23.
- Yin S, Wang X, Shi M et al. Improvement of oxytetracycline production mediated via cooperation of resistance genes in *Streptomyces rimosus*. *Sci Chin Life Sci* 2017;**60**:992–9.
- Zakrzewski P, Medema MH, Gevorgyan A et al. MultiMetE-val: comparative and multi-objective analysis of genome-scale metabolic models. *PLoS One* 2012;**7**, DOI: 10.1371/journal.pone.0051511.
- Zani CL, Carroll AR. Database for rapid dereplication of known natural products using data from MS and fast NMR experiments. *J Nat Prod* 2017;**80**:1758–66.
- Zhang B, Zhou YT, Jiang SX et al. Amphotericin B biosynthesis in *Streptomyces nodosus*: quantitative analysis of metabolism via LC-MS/MS based metabolomics for rational design. *Microb Cell Fact* 2020;**19**, DOI: 10.1186/s12934-020-1290-y.
- Zhang JJ, Tang X, Moore BS. Genetic platforms for heterologous expression of microbial natural products. *Nat Prod Rep* 2019;**36**:1313–32.
- Zhang L, Zhao G, Ding X. Tandem assembly of the epothilone biosynthetic gene cluster by in vitro site-specific recombination. *Sci Rep* 2011;**1**:1–5.
- Zhao Q, Wang L, Luo Y. Recent advances in natural products exploitation in *Streptomyces* via synthetic biology. *Eng Life Sci* 2019;**19**:452–62.
- Zhou Q, Ning S, Luo Y. Coordinated regulation for nature products discovery and overproduction in *Streptomyces*. *Synth Syst Biotechnol* 2020;**5**:49–58.
- Zhou Z, Gu J, Du Y-L et al. The -omics era- toward a systems-level understanding of *Streptomyces*. *Curr Genomics* 2011;**12**:404–16.
- Zhou Z, Sun N, Wu S et al. Genomic data mining reveals a rich repertoire of transport proteins in *Streptomyces*. *BMC Genomics* 2016;**17**:510.