Chapter

Streptomyces as a Source of Antimicrobials: Novel Approaches to Activate Cryptic Secondary Metabolite Pathways

Ángel Manteca and Paula Yagüe

Abstract

Streptomyces is the most important bacterial genus for bioactive compound production. These soil bacteria are characterized by a complex differentiation cycle. *Streptomyces* is extremely important in biotechnology, producing approximately two thirds of all antibiotics, as well as many compounds of medical and agricultural interest. Drug discovery from streptomycetes became challenging once the most common compounds were discovered, and the system was basically abandoned by industry. Simultaneously, antibiotic resistance is increasing dramatically, and new antibiotics are required. Screening from nature is being resumed (exploring new environments, looking for elicitors, metagenome, etc.). Secondary metabolism is conditioned by differentiation; although the relationship between both has long remained elusive, differentiation as a trigger for antibiotic production remains basically unexplored. Most cultures used in screening campaigns for new bioactive molecules have been performed empirically, and workflow was extremely productive during the so-called golden age of antibiotics; however, currently there is a bottleneck. *Streptomyces* is still the most important natural source of antibiotics, and it also harbors many cryptic secondary metabolite pathways not expressed under laboratory conditions. In this chapter, we review strategies based on differentiation, one of the keys improving secondary metabolite production and activating cryptic pathways to face the challenges of drug discovery.

Keywords: *Streptomyces*, screening, antibiotics, secondary metabolism, differentiation, elicitors, morphology, liquid cultures

1. Introduction

1.1 *Streptomyces* development

1.1.1 Solid cultures

The complex *Streptomyces* life cycle on solid-grown cultures is made up of different phases.

Microorganisms on soils are exposed to different stress factors: chemical, physical and/or biological. Of these variable conditions, sometimes nutrients can

be limited, preventing growth [1]. A good survival strategy of some bacteria and fungi is the latency stage of spores. Under this stage, cells stop growing and their metabolism is mainly inactive [2]. Spores are very resistant to stress conditions. In Streptomycetes, they are formed by thick hydrophobic covers that preserve genetic information during unfavorable conditions. Spores are dispersed by wind, water or insects and remain in latency until germination in favorable situations [3]. Besides the thickness of the cell wall, their resistance to extreme temperatures and other physicochemical factors is because of their low water content [4]. In this dehydrated environment, molecules have inactive conformations (denaturalized proteins, etc.) that are stabilized and protected by the sugar trehalose [2]. From this null or limited metabolism, spores are able to germinate, as the macromolecules needed have already been synthesized before the latency stage [2]. For germination, optimal hydration conditions are required. However, it can be faster and more successful in the presence of nutrients and specific stimuli. Spores also need enough levels of intracellular trehalose [5], and polyphosphates [6] that allow metabolic activity to commence before cells are able to detect and assimilate an external source of energy [2].

Despite the fact that germination is a very important stage in the *Streptomyces* life cycle, it has remained largely unstudied, because it is not an industrially important stage. When environmental conditions are prosperous, spores on soils/laboratory conditions (petri plates with solid culture medium) germinate. Germination of *Streptomyces* spores is a sequential process, divided into at least four stages: darkening, swelling, emission of the germination tube [7] and reduced swelling [8].

Darkening is the stage in which spores allow the entry of water into the cell losing their impermeability and heat resistance. After a few minutes, spores change their optical characteristics and the cells are able to reactive their metabolism. Darkwelling requires bivalent cations such as Ca^{+2}, Mg^{+2}, Mn^{+2}, Zn^{+2} and Fe^{+2} as well as energy reserves of the spore [7, 9, 10]. Degradation of the cover leads to the reactivation of the cell wall hydrolases. These enzymes, like Rpf (Resuscitation-promoting factors), are involved in cell wall degradation, facilitating peptidoglycan remodeling by hydrolyzing glycosylic bonds B-1,4 between cell wall components, N-acetylglucosamine and N-acetylmuramic acid, which in turn, allow the access to external nutrients [2].

Swelling is the stage in which spores increase its volume due to the entrance of water. This increases the levels of glucose, as a result of trehalose hydrolysis [11]. This seems to be an essential step in germination since only when trehalose concentrations decrease and proteins able to recover their functional conformations [12, 13]. Ribosomes are inactive inside the spores [14, 15]. However, only few minutes after germination starts (30–60 min), they are fully functional and new proteins can be translated [6]. During this stage, spores are metabolically active and are able to use trehalose as main energy source [11].

Emission of the germination tube: This stage begins at the same time as the first DNA replication, when spores are able to detect external sources of nutrients and adjust their metabolic pathways as a result [2]. The accurate point from which the germination tube is emitted is indicated by the accumulation of the protein, SsgA [3]. SsgA is not essential for germination; nevertheless, its absence significantly reduces the number of germ tubes in each spore, and its overexpression has the opposite effect [3].

Reduced swelling: Once the germ tube emerges, the spore starts to gradually reduce its swelling. This stage depends on the quantity of peptidoglycan cross-link that involves a carboxypeptidase (which corresponds to the SCO4439 gene in the *Streptomyces coelicolor* genome www.strepdb.com). A deletion of this

carboxypeptidase causes spores to continue swelling until 5 μm diameter (2.5 times more than the wild-type strain), after the emission of the germ tube [8]. The existence of this stage demonstrates that the regulation of water entering into the spores is complex and highly regulated.

During germination, several important morphological changes occur, including the degradation of the spore cover, which is mainly formed of peptidoglycan, and causing a huge increase of metabolic activity [2]. On the other hand, shortly after the emission of the germ tube, the expression of the protein DivIVA starts [6]. This protein plays a critical function in the vegetative growth and is located at the apex of the hyphae [16–18]. After germination, genes involved in cell division (like *ftsZ*) and proteins involved in cellular growth (like FilP) are expressed [2]. The expression of these genes is considered as the final of the germination phase [2, 6].

Curiously, during germination, three different secondary metabolites are produced: albaflavenone (a terpenoid) with an antibacterial effect against *Bacillus subtilis*, giving an important advantage in competitive environments, and two polyketides (germicidin A and chancone) with a germination inhibitory effect. These compounds are *de novo* generated during germination and are good examples of compounds produced during vegetative growth [19].

After germination, the *Streptomyces* mycelium starts to grow apically (regulated by *div*IVA gene) as a very transitory, multinucleated stage called first mycelium (MI), which is multinucleated [20]. This is a very transitory stage in which dead and living segments alternate in the same hyphae [21]. There are two kinds of septa separating segments during the MI stage: one of them is composed of a cell membrane and cell wall (with peptidoglycan) and the other, recently discovered, is a 1-μm spacing cross-membrane-based septa, without peptidoglycan [20]. *Fts*Z participates in this septation, but it is not essential, as cross-membranes were observed in the *Fts*Z null mutant. Later, the dead segments are completely disintegrated and living segments continue apical growth. In this phase, the growth is especially branched [18].

After this early stage of branching growth, there is a morphological differentiation into a second mycelium (MII), a sort of multinucleated mycelium with sporadic and apparently randomly positioned septa (formed of cell membrane and cell wall), which is the secondary metabolite producer [22]. This mycelium has two different populations: one of which remains as a branching mycelium growing on surfaces and is called substrate mycelium and the other develops hydrophobic covers and starts to grow into the air without branching (aerial mycelium) [18]. This is known as the "sky-pathway," which regulates the expression of genes encoding proteins forming hydrophobic proteins (rodlins, chaplins and ramS) that cover the aerial hyphae. Some of the so-called "bald" (*bld*) mutants among others (defective in aerial growth) regulate the activation of this pathway [23]. At the end of the cycle, aerial hyphae undergo extensive synchronous septation (cell division) to create up to 100 uninucleoid compartments, which finally differentiate to create chains of spores [24]. At the end of the cycle, the "white" (*whi*) genes are activated (whose mutants are defective in the formation of mature gray spores on the fluffy aerial mycelium) and the hyphae septate and sporulate [25].

1.1.2 Liquid cultures

Streptomyces differentiation in liquid cultures has hardly been studied, mainly due to the fact that most strains do not sporulate under these conditions. However, the fact that *Streptomyces venezuelae* is capable of sporulation in liquid cultures has made it a new study model in recent years [26].

The applications of novel methodologies (confocal microscopy, proteomics, transcriptomics) to the study of *Streptomyces* biology have extended the knowledge regarding the development during the phases preceding sporulation in liquid cultures [27–31].

In these conditions, in most *Streptomyces* strains, aerial mycelium formation and sporulation are blocked [28]; so, there is no hydrophobic cover formation, or sporulation, but there is a first exponential growth phase corresponding to the compartmentalized MI morphology, and then there is a growth arrest phase corresponding to programmed cell death (PCD). After that, the hyphae, which remain alive, continue growing in a second exponential growth phase in which the morphology of the hyphae becomes MII multinucleated mycelium, which corresponds to the antibiotic production [27, 30].

1.2 Correlation between *Streptomyces* life cycle and antibiotic production

Most industrial processes for secondary metabolite production are performed in liquid (flasks or bioreactors). This fact makes the knowledge of the *Streptomyces* behavior essential in these conditions. The optimization of *Streptomyces* liquid cultures has been empiric for decades; however, the end of the so-called "golden age of antibiotics," which meant fewer and fewer compound discoveries or the advances in genome sequences revealing the existence of cryptic biosynthetic pathways that are not expressed under laboratory conditions, calls the attention of the scientific community.

Work on *Streptomyces* differentiation in liquid cultures has largely focused on the analysis of mycelial macroscopic morphology (pellets/clumps formation), media composition and bioreactor design [31]. The differentiation of the hyphae during the life cycle is essential for secondary metabolism [30]; however, there is still some controversy over which is the best morphology for production. Some authors affirm that pellets are better for production, while others report about clumps or even disaggregated growth. Hence, these conditions seem to be strain dependent. For instance, in *Streptomyces olidensis* (retamycin) [32], *Streptomyces tendae* (nikkomycins) [33], *Streptomyces lividans* (hybrid antibiotics) [34] and *Streptomyces coelicolor* (Undecylprodigiosin, Actinorhodin) [30], pellet formation is essential for good production. However, in *Streptomyces noursei* (nystatin) [35] and *Streptomyces fradiae* (tylosin) [36], formation of pellets leads to worse antibiotic production than disaggregated growth. The lack of a reliable developmental model in streptomycetes liquid cultures has hindered the precise identification of reliable phenotypes for use in the analysis and optimization of industrial fermentations. It has been demonstrated that antibiotics are produced by the substrate mycelium at the end of the proliferation phase, i.e., second mycelium morphology (after PCD process) [27, 30, 37]. However, despite the fact that there is a distinctive mycelium producing antibiotics (MII) [27, 30] (hence there is not secondary metabolite production until the differentiation of MII), antibiotic production has additional regulations, and each *Streptomyces* strain does not display its entire potential secondary metabolism at a specific developmental condition.

1.3 Screening for new bioactive compounds and drug discovery approaches

Most antibiotics and other bioactive compounds (antitumorals, immunosuppressors, etc.) were discovered from actinomycetes, but this source of drugs became challenging once the most common compounds were discovered. New antibiotic scaffolds are required, and resuming screening from nature is mandatory.

During the past 30 years, only two new classes of antibiotics have been brought to the clinic for treatment systemic infections [38]. At the same time, microbial resistance to existing antibiotics has increased dramatically, rendering some microbial infections that are extremely hard to treat. The current scenario looks gloomy, and there is a concrete risk of humanity returning to the pre-antibiotic era, with a high mortality from routine surgical or chemotherapeutic procedures because of infections by antibiotic-resistant pathogens. There is a general consensus that new antibiotics are urgently required and are the best chance in the fight against resistance.

Only a fraction of secondary metabolite pathways is active in actinomycete laboratory cultures, and there are a large number of strains whose potential to produce bioactive compounds remains unexplored [39]. When whole genome sequencing became available at the start of the twenty-first century, the existence of many silent or cryptic biosynthetic gene clusters in actinomycetes was revealed, which may encode antibiotic-like substances but are not, or only poorly, expressed under laboratory growth conditions. Each *Streptomyces* strain harbors 20–50 biosynthetic pathways for natural products, for which only some products have been identified [39]. The big challenge is to find ways to activate these pathways so as to allow screening for new secondary metabolites from nature to resume.

2. Strategies in drug discovery

Once the most common antibiotics and bioactive compounds were discovered, drug discovery has become more challenging, and industry had mostly discontinued the search for new drugs, with *Streptomyces* as the source. Nevertheless, the urgent necessity of new antibiotics in the clinic has caused screening from nature to be resumed [39]. New environments are being explored and some new actinomycete strains have been discovered [40]; the potential of the marine environment has been highly explored during last decade, as well as some, *a priori* hostile soils, such as high mountains, deserts or icy places. The scientific community is also paying more attention to symbiotic relationships.

Thinking of natural conditions of growth is another important point of view. Laboratory/industrial conditions in which microorganisms are typically grown are very different conditions from nature, which imply the dormancy of most of the genetic pathways involved in antibiotic production, since these substances are related to stress conditions or defense against niche competitors. Furthermore, it is necessary to take into account that the majority of environmental bacteria are not easily culturable or are even nonculturable in laboratory conditions. This broad view is now possible, thanks to the access to the metagenome in different environments [41].

Another current strategy is to look for elicitors. Elicitors are small molecules, which are able to induce *Streptomyces* differentiation as well as trigger cryptic antibiotic pathways.

Additionally, secondary metabolism is conditioned by differentiation, so it is another aspect that requires special attention. As mentioned above, the life cycle stages and the mycelium morphology are keys for antibiotic production.

At present, different complementary strategies are being developed to improve secondary metabolite production and active cryptic biosynthetic gene clusters. One way to classify them is by unselective and selective methods [42], considering unselective methods are used in the improvement of screening of new activities and selective methods are used in the improvement of production of known molecules.

2.1 Methods for searching for new bacteria/compounds

2.1.1 Exploring new environments

The actinomycetes are widely distributed in the environment; they were discovered at the end of the nineteenth century as a group of soil-living microorganisms. However, their importance started in 1943 with the discovery of streptomycin, the first effective treatment against tuberculosis by the most important actinomycetes genus, *Streptomyces*. Researchers have isolated different *Streptomyces* as soil inhabitants during decades as the most important source of antibiotics and bioactive compounds in nature. Clavulanic acid (*Streptomyces clavuligerus* [43]), neomycin (*S. marinensis* [44]), chloramphenicol (*Streptomyces venezuelae* [45]), the insecticide avermectin (*Streptomyces avermitilis* [46]), the immunosuppressant tacrolimus (*Streptomyces tsukubaensis* [47]), kanamycin (*Streptomyces kanamyceticus* [48]) and potent antitumoral platenolides (*Streptomyces platensis* [49]) are only few examples of the 12,400 bioactive compounds produced by the genus *Streptomyces* that society is routinely using in the clinic or agriculture. The rest of the phylum produces 3600 bioactive compounds (being 3400 antibiotics) [50]. Until the 1980s, new bioactive compounds were discovered relatively easily; however, since 1985, only three new compounds have been discovered, with the last one, platensimycin (*S. platensis* [40]), in 2006.

Once the most common compounds are discovered and producer strains are isolated, one of the strategies for founding new activities is the exploration of new environments, under-exploited habitats, generally difficult to access. Marine environment are an important niche of new species of *Streptomyces* [51]. Besides marine actinomycetes, they have been isolated from Himalaya Mountains [52], several islands [53], Atacama Desert [54], Antarctica [55] and several extremophile habitats [56–58].

Promising results and new species are being discovered from these unexplored areas, in combination with next-generation genome sequencing, metagenomics and new ways of bacterial culture [59], being one of the most important approaches in the new age of bioactive compound research.

2.1.2 Symbiosis relationships

As mentioned above, *Streptomyces* are ubiquitous in soils. However, it has been discovered that they are not only free-living soil bacteria but many have also evolved to live in symbiosis with plants, fungi and animals [60]. Their secondary metabolism is a consequence of these complex interactions [60].

There are many and very diverse symbiotic relationships, which involve *Streptomyces*. One of the first discovered is its relationship with plant roots providing a natural defense against fungal infections [61]; it is very common to find bacteria of the *Streptomyces* genus in the rhizosphere. This fact raises the possibility, currently discussed, about its use as biofertilizers in crops [62, 63]. Furthermore, genome mining is now allowing us to know on one hand which genes are involved in this interaction and on the other hand the discovery of potential novel molecules produced only in these conditions [64].

Other important interactions of *Streptomyces* genera take place between bacteria and insects. For instance, in the case of Beewolf digger wasps, *Streptomyces* provides protection against pathogens to the larval cocoon producing a mixture of nine antibiotic compounds [65, 66].

In *Streptomyces* isolates from colonies of the South African termite *Macrotermes natalensis*, a number of interesting novel compounds including natalamycin A

[67] and the potent antitumor geldanamycin [67] have been discovered. Another *Streptomyces* isolate from the same termite produces a novel cyclic analogue of dentigerumycin [68], the antifungal from the fungus-growing ant system. Finally, a series of glycosylated isoflavonoid compounds, including termisoflavone A [69], were recovered from a third *Streptomyces* isolate.

In 2012, 30 *Streptomyces* strains were isolated from a different South African fungus-growing termite, *Microtermes* sp., and two novel polyketides, microtermolides A and B [69], were identified.

Two *Streptomyces* strains, isolated from *Dendroctonus frontalis* beetles, inhibit the antagonistic *Ophiostoma* fungus, but not the mutualistic fungus *Entomocorticium*, suggesting a defensive role that supports the beetle-fungus mutualism. The molecules responsible for this selective antifungal activity are the polyketide mycangimycin [70] and the polycyclic tetramate macrolactams, frontalamides A and B [71, 72].

Other important symbiotic relationship takes place between ants and *Streptomyces*. In recent years, new strains and compounds have been isolated from ant head, legs and nests [73–76].

2.1.3 Nonculturable microorganisms

Since the beginning of microbiology, it was known that many microorganisms do not grow under laboratory conditions and we cannot cultivate them. The new techniques of massive sequencing have revealed the existence of a huge amount of nonculturable microorganisms. Nonculturable microorganisms are, in fact, a majority, and they represent an important challenge in the screening for new secondary metabolites. Next generation sequencing (NGS) has revealed the big pharmacological potential of uncultured bacteria; hence, approaches to improve cultivation-based methods, such as isolation chip (iChip), which is a method of culturing bacteria within its soil environment, or co-cultures, culturing actinomycetes with other bacteria (generally species with which they naturally coexist), are being used successfully [70–72]. The combination of iChip and unexplored ecosystems or symbiotic relationships, mentioned above, is promising as well.

2.2 Methods for screening improvement in a known microorganism (unselective)

Unselective methods include classical strategies for improving the production of a certain compound by modifying the growth of the *Streptomyces* strain. This strategy used to be empiric.

2.2.1 Changing media components

Streptomyces grow well in rich culture mediums; nevertheless, antibiotic production often needs some specific nutrients. Sometimes defined medium can be better for determined compounds. Optimization of nutritional requirements has been one of the most useful changes for secondary metabolite activities [73–76].

2.2.2 Inducing stress response

Stressful situations are one of the reasons why microorganisms start to produce and export bioactive compounds. For this reason, the induction of cultures with several treatments of stress is one of the improvement strategies for synthesis of secondary metabolite compounds in bacteria. Heat and ethanol shock treatments,

high salt conditions, increased hydrostatic pressure, acidic pH shock or feed limitations are some examples of this classical strategy (reviewed in [77]).

2.2.3 Random mutagenesis

There are different random mutagenesis methods used in *Streptomyces*: chemical mutagenesis, in which are used (e.g., by N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), ethyl methanesulfonate (EMS), or nitrous acid (NA)) and physical mutagenesis (e.g., by ultraviolet (UV) light or X-rays)) that remain being robust methods to generate very high-producing strains [78–80].

On the other hand, there is transposon mutagenesis, a powerful tool for random mutagenesis of bacterial genomes and insertion of foreign DNA. A plasmid containing the enzyme transposase and the transposon (small fragment of DNA with transposase target regions) is extracted and inserted randomly into the host chromosome. Transposition creates single mutations that can be identified and mapped by plasmid rescue and DNA sequencing, generating a direct link between the higher yield (phenotype) and the transposon insertion (genotype) [81, 82].

2.2.4 Ribosomal engineering

The fact that some antibiotic resistances trigger some cryptic pathways of secondary metabolites has been recently used as another strategy in the search for new compounds.

It has been demonstrated that this occurs in strains affected by antibiotics, which target a ribosomal protein (for instance, streptomycin, paromomycin and gentamicin [42]), so the effects of the mutations at this level have had very good results, for example, in *Streptomyces coelicolor*, for improving secondary metabolite production [83]. The mechanism of this strategy is still unknown, but it has been proved that there is activation of the positive regulators like *act*II-*orf*4 gene at least in *S. coelicolor* [42]. Through this approach, the antibacterial piperidamycins family compounds have been discovered [84].

2.2.5 Elicitors

Elicitors are small diffusible molecules, which are being used as signals for improving secondary metabolite production or even activating cryptic antibiotic pathways [85].

Elicitors are also present in *Streptomyces* natural environments; hence one of the strategies for stimulating bioactive compound production is the co-culture of different bacteria [85, 86]. Co-cultures usually include species that have symbiotic relationships with *Streptomyces* in nature [87, 88] or pathogenic partners to activate the production of antimicrobial compounds against them [71, 89, 90].

Other kinds of elicitors are also useful, for instance, fungal elicitors (a complex mix of fungal cell walls and filtered fungi cultures) can positively affect the production of natamycin [91]; small molecules, such as GlcNac or phosphate, can activate differentiation and antibiotic production in *S. coelicolor* through the activation of *actII*-ORF4/*red*Z genes in some conditions [92].

At the end of the 1980s, the discovery of new compounds reached a bottleneck, and screening processes often led to the rediscovery of compounds already known. To avoid rediscovery, Pimentel-Elardo et al. [93] developed a workflow based on the use of chemical elicitors combined with activity-independent screening [93]. Activity-independent screening is based on the low-abundant

compounds found in biological samples. The use of elicitors increases the production of these low-abundant compounds, distinguishing them from the most abundant ones. The elicitor "CI-ARC" has been identified as being responsible for triggering several cryptic biosynthetic genes [93].

2.2.6 Differentiation strategies based on morphology

2.2.6.1 Streptomyces behavior in liquid cultures

Large-scale antibiotic production is mostly performed in liquid cultures. Macroscopic morphology of the mycelium (pellets and clumps) correlates with the production of secondary metabolites [30]. The genes controlling mycelium aggregation have been characterized in the *S. coelicolor mat* gene cluster [94], and the *cslA, glxA* and *dtpA* genes [95–97] are responsible for pellet formation. This knowledge can be used for controlling and improving morphology in industrial fermentations.

Since spores are inoculated in liquid medium, there is an aggregation trend [98]. Germlings determine the macroscopic morphology (pellets and clumps) of the culture, which involves the highly conserved genes *matA, matB* [94, 98] and the *cslA/glxA* operon [98].

In liquid cultures, there are some strains able to sporulate [99], but even those which aren't able to sporulate in flasks can sporulate in bioreactors, beacuse of the stess conditions inside the fermenter, such as lack of oxygen, strong agitation, etc. [100]. When *Streptomyces* bacteria sporulate, all the metabolic efforts are focused on that, which means that the secondary metabolism stops by. Therefore, one of the main things for taking into account during a screening for new secondary metabolites is to avoid sporulation in the industrial fermentations. Hence, it is very important to look out the cultures for keeping secondary metabolism as much as possible [100].

Morphology in liquid cultures can be monitored by several tools. Laser diffraction has been used to measure pellet size, an improvement over image analysis, which is trickier and requires more time [101], and a technique based on flow cytometry has been used to establish the pellet size distribution of a culture population [102, 103]. Recently, a useful algorithm has been developed as a plugin for the open-source software ImageJ to characterize the morphology of submerged growing cells [104]. On the other hand, mathematical models have been performed to predict the behavior of *Streptomyces* liquid cultures based on pellet/clump morphology [105, 106].

Furthermore, it has been reported that the oxygen transfer rate (OTR) and oxygen uptake rate (OUR) are crucial for the development and production in liquid cultures [100]. Controlling these rates, which directly affects morphology, was described as being crucial for scaling up production to industrial conditions [107]. Biophysical parameters, including pH, viscosity, agitation, dissolved oxygen levels and surface tension, directly affect mycelium morphology. The optimization of these factors is another factor to consider [31].

2.2.6.2 Programmed cell death and MII differentiation

As mentioned in the introduction of this chapter, programmed cell death (PCD) is a key event, triggering the differentiation of antibiotic producer mycelium (MII) in liquid and solid cultures [30]. However, the specific signals derived from cell death are not yet known. The N-acetylglucosamine resulting from

peptidoglycan dismantling accelerates the development and antibiotic production [108, 109] and might be one of the signals released during PCD. A simple methodology based on fluorimetric measures was designed to quantify PCD in liquid cultures [37], allowing prediction of the efficiency of antibiotic production based on the level of PCD.

PCD and MII differentiation can be modulated by modifying culture conditions and morphology. Modifying developmental conditions to enhance PCD and MII differentiation leads to the improvement of secondary metabolite production. This approach was recently applied in a study combining the heterologous expression of plant flavonoids in *Streptomyces albus* with a strategy to enhance PCD and MII differentiation [110]. The same strategy was successful at improving mTGase production from *Streptomyces mobaraensis* [111]. Therefore, PCD and MII differentiation were demonstrated to be crucial for the streamlining of secondary metabolite production in bioreactors [100].

2.3 Methods for production improvement in a known compound (selective)

Selective methods with a specific target include regulatory engineering, heterologous expression, genome mining and combinatorial biosynthesis.

2.3.1 Regulatory engineering

Each active compound, for example, antibiotic or antitumor, etc., has several molecules synthesized first in the pathway, and sometimes these molecules are a limited source for the compound of interest. In this sense, one strategy widely used is the addition of these precursors, previously synthetized from an external source. In the same way, it is possible to enhance the production of a specific compound through the overexpression of its positive regulators. Inversely, knocking out the suppressors (negative regulation) of the gene of interest has also had good results increasing the final compound production [112, 113].

2.3.2 Heterologous expression

In microbiology, this molecular genetic technique is based on the expression of a gene or a group of genes (gene cluster) in a host microorganism that does not have this particular gene or gene cluster in its own genome. This procedure, performed by recombinant DNA technology, is very useful when hosts are bacteria with simple developmental cycles, such as *E. coli* or *Bacillus* [114]. However, in the case of *Streptomyces,* due to its complex pathways to produce antibiotics, heterologous expression usually works only among *Streptomyces* strains [115, 116], not solving the problem of its tricky growth.

Nevertheless, good results have been achieved in activating cryptic metabolites, for example, by using the widely conserved *Streptomyces coelicolor* pleiotropic regulator *Afs*Q, which activates the production of new compounds in several streptomycetes [117]. Despite the fact that heterologous expression in *Streptomyces* is a challenge, this technique continues to be improved [118].

2.3.3 Genome mining

Genome mining is defined as the obtaining of information about an organism, based on genome analysis. In the postgenomic era, the combination of easier genome sequencing and bioinformatics analyses allows researchers to uncover the

biosynthetic potential of the microorganisms, i.e., the gene clusters for producing secondary metabolites into actinomycetes strains that are in the genome, but silent. Genome mining is nowadays one of the more powerful tools in the screening for new antibiotics [119–122].

2.3.4 Combinatorial biosynthesis

Combinatorial biosynthesis can be defined as the application to modify biosynthetic pathways to natural products to produce new and altered structures using nature's biosynthetic machinery [123]. The chemical modification of existing chemical scaffolds is challenging and sometimes provides only a temporary solution against resistant organisms [124]. Combinatorial biosynthesis has been largely developed over the last 20 years, and it has been enhanced, thanks to genome mining and synthetic biology [125–127].

3. Conclusions

Humanity faces the great challenge of fighting antibiotic resistance, which is growing at a faster rate than our capacity to find new antimicrobials and new strategies to solve this problem.

The *Streptomyces* genus is still a huge source of natural bioactive compounds, but we need to elaborate new multidisciplinary strategies to avoid rediscovering the same compounds. Therefore, we need to invest efforts into developing alternative strategies to resume screening from natural actinomycetes.

There is not a "magic" methodology to activate cryptic pathways and improve the discovery of new bioactive compounds, but multidisciplinary approaches combining the methodologies discussed in this chapter will play a key role in the improvement of screening for new bioactive compounds from streptomycetes.

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Conflict of interest

There is no conflict of interest in this work.

Author details

Ángel Manteca[1] and Paula Yagüe[2*]

1 Oviedo University, Oviedo, Spain

2 Leiden University, Leiden, Netherlands

*Address all correspondence to: p.yague.menendez@biology.leidenuniv.nl

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