Tools for metabolic engineering in Streptomyces

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During the last few decades, Streptomyces have shown to be a very important and adaptable group of bacteria for the production of various beneficial secondary metabolites. These secondary metabolites have been of great interest in academia and the pharmaceutical industries. To date, a vast variety of techniques and tools for metabolic engineering of relevant structural biosynthetic gene clusters have been developed. The main aim of this review is to summarize and discuss the published literature on tools for metabolic engineering of Streptomyces over the last decade. These strategies involve precursor engineering, structural and regulatory gene engineering, and the up or downregulation of genes, as well as genome shuffling and the use of genome scale metabolic models, which can reconstruct bacterial metabolic pathways to predict phenotypic changes and hence rationalize engineering strategies. These tools are continuously being developed to simplify the engineering strategies for this vital group of bacteria.

Introduction

Streptomyces are versatile, gram positive, rod shaped bacteria that form the largest genus in the phylum Actinobacteria.1 These bacteria are soil saprophytes and have large genomes of ~8–10 MB with an extremely high GC content of 70% or greater.2 Their genomes consist of large linear chromosomes and uniquely mainly linear plasmids.3

Streptomyces are very well known for their ability to synthesize many secondary metabolites.2 Among actinomycetes, around 2400 unique compounds are produced by Streptomyces species and they are therefore known as the industrial source of secondary metabolite production.4,5 Each of these secondary metabolites are normally encoded by large 20–30 biosynthetic gene clusters,6,7 which can reach up to 100 kb in size.8,9 These gene clusters are the main targets for improvement of the respective secondary metabolite production. A variety of their high-value commercial products are produced on an industrial scale by microbial fermentation. There has been a constant desire in the fermentation industry for strains with improved production properties.10

Methods of increasing the productivity of industrial microorganisms range from the classical random mutagenesis to the use of more rational methods. One of these rational methods is metabolic engineering where, in order to maximize product yields, primary metabolic fluxes are redirected toward the target and reduces the byproducts. This is performed by the introduction of genetic modifications through recombinant DNA technology, in a manner that supports high secondary metabolite productivities.11,12

Recent advances in the development of modern technologies such as second and third generation sequencing, genomics, proteomics, metabolomics, transcript profiling, and transcriptomics has allowed for more efficient ways to engineer microorganisms for secondary metabolite production.13

This review aims to focus on the tools for metabolic engineering of Streptomyces bacteria for the production of useful secondary metabolites in the past 10 years. In addition, it aims to provide examples of industrial secondary metabolite production and discuss advantages and limitations of the genetic tools and techniques that have been used to engineer higher productivity of these products.

Streptomyces Strains and Their Products

The discovery of Streptomyces as efficient secondary metabolites producers began in the early 1940s with the discoveries of actinomycin and streptomycin in 1940 and 1943, respectively.14 Streptomyces produces about two-thirds of naturally occurring antibiotics and a wide array of other secondary metabolites, including anthelmintic agents, antitumor agents, antifungal agents, and herbicides.5 The production of these secondary metabolites and their essential functions highlights the importance of their production on a large scale.15

The most well studied Streptomyces species are Streptomyces coelicolor and Streptomyces lividans. The work on S. coelicolor has been pioneered by Sir David Hopwood, who began his work on the bacteria in 1950. He discovered that the bacteria produce a striking blue pigment under alkali conditions, when grown on agar media. Its pigmentation characteristic can be exploited for their use as phenotypic markers in genetic studies.16 His work continued further into 1997 when the S. coelicolor genome sequencing project began, with the full genome sequenced by 1999 and published three years later.6 S. lividans is the preferred heterologous host for secondary metabolite production. The main reasons for this include its relaxed restriction-modification system
which allows for the uptake of DNA and its considerably reduced endogenous protease activity which allows for better product recovery.\textsuperscript{16} The physical map of this bacterium was completed by Leblond and colleagues with a genome size of 8.19 Mb.\textsuperscript{17} \textit{S. coelicolor} and \textit{S. lividans} are two closely related laboratory strains that have been used extensively in the development of \textit{Streptomyces} genetic tools. The 16S rRNA sequences of \textit{S. coelicolor} and \textit{S. lividans} share a greater than 99.5\% similarity.\textsuperscript{16} Apart from being so well characterized, they are also easily genetically modified which makes them optimal targets for genetic engineering. More specifically, they may also be used as heterologous hosts for the expression of other genes producing different secondary metabolites.\textsuperscript{2}

The main types of secondary metabolites produced by \textit{Streptomyces} are antibiotics, antifungal agents,\textsuperscript{18} food preservatives,\textsuperscript{19} anthelmintic agents, and antitumor agents.\textsuperscript{18} Each of these categories of metabolites is important in either the medical or food industries and requires industrial scale production to cater for the growing market. For this reason, the \textit{Streptomyces} species that produce them may need to be engineered.\textsuperscript{11}

Recently, Lucas et al.\textsuperscript{5} generated the largest and most comprehensive database of natural products isolated from \textit{Streptomyces} to date. This database is known as StreptomeDB (http://www.pharmaceutical-bioinformatics.de/streptomedb/) and contains >2400 different products isolated from >1900 different \textit{Streptomyces} strains and substrains. The database provides the names, molecular structures, biological role, and synthesis routes of all the products as well as brief descriptions of the organisms and references to scientific literature. This database provides an excellent stepping stone for finding \textit{Streptomyces} compounds as targets for engineering strategies (Fig. 1).

\section*{Streptomyces Engineering Toolbox}

Genetic engineering involves several steps to which tools may be selected or designed in order to perform an informed and targeted genetic change. The first key strategy is to choose clinically relevant secondary metabolites that are amenable to engineering. This amenability is usually attributed to the host organism. The general steps in genetic engineering involve: (1) choosing or developing suitable gene transfer methods, expression hosts, and methods to stably maintain and express the engineered biosynthetic gene(s); (2) cloning and sequencing the biosynthetic gene(s) responsible for the production of the secondary metabolite; and (3) using efficient tools to engineer the biosynthetic pathway.\textsuperscript{20}

A crucial prerequisite for genetic engineering is efficient DNA transfer techniques for recombinant genetic material. Due to the fact that \textit{Streptomyces} are gram-positive bacteria, standard DNA transfer methods established for gram-negative bacteria such as \textit{Escherichia coli} are not applicable and new experimental methods had to be developed. One major difference between \textit{E. coli} and \textit{Streptomyces} is the difference in their cell walls. A requirement for transformation in \textit{Streptomyces} is the formation of protoplasts to increase the ease of DNA transfer techniques by the removal of their thick cell wall.\textsuperscript{21}

Four different techniques were established to transfer recombinant DNA into \textit{Streptomyces} cells in an efficient manner. Two of these DNA transfer techniques are used regularly and include: (1) transformation with plasmid, cosmid, or chromosomal DNA and (2) conjugation between \textit{Streptomyces} or intergenic conjugation from \textit{E. coli}.\textsuperscript{21}

Transformation involves the uptake and incorporation of exogenous genetic material to an organism of interest using either chemical or physical means.\textsuperscript{21} Bibb et al.\textsuperscript{22} found that chemical transformation of protoplasts in \textit{Streptomyces} was effective with the addition of polyethylene glycol (PEG). The protocol outlining the transformation procedure can be found in Practical \textit{Streptomyces} Genetics.\textsuperscript{23} The transformation efficiencies of \textit{Streptomyces} vary depending on the species and type of molecule used. Using the two model organisms, \textit{S. lividans} and \textit{S. coelicolor}, approximately $10^5$–$10^7$ transformants per microgram of DNA can be expected with plasmids up to 60 kb in size. Electroporation uses the addition of a short electric current to create transient pores in the membrane for the uptake of DNA. The advantage of this method over chemical
transformation is there is no need for protoplast formation and regeneration. However, conditions for electroporation are strain specific, and therefore optimization is required based on the *Streptomyces* species.21

Conjugation involves the transfer of genetic material through direct cell-to-cell contact. Intergeneric conjugation between *E. coli* and *Streptomyces* is possible with the use of bi-functional vectors, which are species specific.23 There are several advantages of using conjugation over transformation as a DNA transfer method: (1) There is no need for protoplast formation and regeneration. (2) Restriction barriers may be surpassed if single stranded plasmid DNA is transferred. (3) Vectors that allow for site specific integration can be used.21

Over the last decade, a variety of cloning vectors has been constructed for genetic engineering of *Streptomycetes*. This is because previous vectors which were used for Gram negative and even Gram positive bacteria were not able to replicate in *Streptomyces*. It would go beyond the scope of this review to describe all of these in detail, but these vectors, which are mainly plasmids, are species specific. In addition, these vectors need to be designed to contain replicons for replication in *Streptomyces* as well as antibiotic resistance genes to act as selectable markers.2, 21

Table 1 shows several common cloning vectors used in *Streptomyces* most recently.

| Plasmid               | Organism                | Method of gene introduction | Reference |
|-----------------------|-------------------------|-----------------------------|-----------|
| pLAE001               | *S. albus*              | Transformation              | 48        |
| pLAE003               | *S. albus*              | Conjugation                 | 49        |
| pIJ702                | *S. coelicolor*         | Transformation              | 50        |
| pIJ703                | *S. lividans*           | Transformation              | 52        |
| pUZ8002               | *S. coelicolor*         | Conjugation                 | 55        |
| pHZ1351 derivatives   | *S. coelicolor*         | Transformation              | 53        |
| pFD666 (cosmid vector)| *S. aureofaciens and S. lividans* | Transformation | 50        |
| SCP2 and SCP2*        | *S. coelicolor and S. lividans* | Transformation | 51        |

**Metabolic Engineering**

Metabolic engineering has been described as the “improvement of cellular activities by the manipulation of enzymatic, transport and regulatory functions of the cell with use of recombinant DNA technology”26. Metabolic engineering considers whole cellular systems and any genetic manipulations would change the efficiency of the overall bioprocess, thus distinguishing it from simple genetic engineering. Metabolic engineering also allows for defined and rationalized changes to render a particular improvement. As opposed to plain genetic engineering, metabolic engineering may change the whole dynamics of a particular pathway to improve its functionality. Metabolic engineering of a variety of microorganisms has been previously reviewed in detail.18, 27

There may be several different motives for the metabolic engineering of microorganisms which can include: (1) Improvement of yield and productivity of a commercially viable product. (2) Extension of the substrate range of a product. (3) Removal or reduction of by-product formation. (4) Introduction of innovative pathways for product biosynthesis.28

Successful metabolic engineering techniques are only applicable with the knowledge of well-characterized biosynthetic pathways and to organisms for which molecular tools are available and optimized to some extent. In addition, all cells have highly interconnected metabolic pathways and any changes to the specific pathway may affect other pathways as well as cell growth and this need to be taken into consideration. It is therefore challenging to pre-evaluate the effect of genetic changes and identify the accurate target genes for efficient strain improvement.29

**Selection of the Method of Genetic Modification**

Metabolic engineering can be achieved through several different approaches which improve the efficiency of the secondary metabolite production pathway. Each of these approaches target different components of a typical metabolic pathway. These include precursor engineering, engineering structural
genes, engineering regulatory networks, and genome shuffling. *Streptomyces* genetics has progressed from genetic engineering to metabolic engineering over the last decade. The necessity for metabolic engineering stems from the complex pathways used to synthesize secondary metabolites as opposed to single isolated genes. Due to this in-depth analysis of the pathways are required followed by the targeting of possibly more than one gene in the pathway.\(^{30}\) Table 2 shows metabolic engineering successes in *Streptomyces*.

Precursors for secondary metabolite production are vital and are often the products produced through the catabolism of carbon compounds during primary metabolism. This means precursors are found at the intersection of primary and secondary metabolism. In order to increase the production of secondary metabolites, the precursor should be readily available and in certain instances this is not the case. The engineering approach is therefore to genetically engineer certain enzymes in order to increase the availability of certain precursors in different pathways.\(^{30}\) One such pathway is the glycolytic (Embden-Meyerhof) pathway, which is involved in the conversion of glucose to pyruvate. The precursor engineering approach was applied to the production of clavulanic acid.\(^{31}\) This was achieved by disrupting of *gap1* and *gap2* genes which code for glyceraldehyde-3-phosphate dehydrogenases. These are involved in the conversion of D-glyceraldehyde-3-phosphate (G3P) into 1,3-diphosphoglycerate (1,3-BPG). G3P is one of the precursors in the biosynthesis of clavulanic acid. The genes were disrupted by means of excision and ligation mechanism in different plasmids. The accumulation of the G3P precursor resulted in an increase in the production of clavulanic acid by 2-fold (Fig. 2). Actinorhodin, a polyketides antibiotic produced by *S. coelicolor*, is another example where increased precursor availability leads to enhancement of product production. Ryu and colleagues\(^{32}\) attempted overexpressing by adding an additional copy on a plasmid. *accA2, accB,* and *ace* genes code for the different subunits of the enzyme acetyl-CoA carboxylase (ACC). These genes are precursors for actinorhodin production. The results showed an enhancement of carbon flux to malonyl-CoA, which is also a precursor enzyme of actinorhodin resulting in a 6-fold increase in the production of the product.

Structural genes found in the metabolic pathways responsible for the production of the secondary metabolites are usually attractive targets for metabolic engineering. In these cases, the gene dose may be increased; the gene may be inactivated, deleted, or modified.\(^{30}\)

Increasing the dose of a particular gene would theoretically correspond to an increase of the target protein and thus increase the production of secondary metabolites. Integration of the clavaminane synthase gene, *cas2* into the chromosome of *S. clavuligerus*, resulted in up to 5-fold increase in clavulanic acid production.\(^{33}\) This was done using recombinant plasmids designed in the study. Plasmids were constructed for both overexpression and different plasmids for chromosomal integration and transformed into *S. clavuligerus* (Fig. 2).

Furthermore, the duplication of *pah2* encoding a proclavaminate amidino hydrolase, has been recently reported to improve clavulanic acid production by introducing a single additional copy of *pah2* introduced into chromosome via an integrative plasmid (Fig. 2).\(^{34}\)

For the increase of Nikkomycin X production, which is an antifungal agent produced in *S. ansuchromogenes*, the gene dose was increased by the duplication of the *sanO* gene, which codes for a non-ribosomal peptide synthetase (NRPS). This was done by producing a recombinant strain using the pWOG plasmid, which contains *sanO*. This lead to a 2-fold increase.\(^{35}\)

Inactivation or deletion of genes can improve metabolite production by removing genes coding for activities that transform the metabolite into a different one. In addition it can also involve the removal of genes whose products inhibit or degrade the target product.\(^{30}\) Inactivation of the *nysF* gene by the insertion of a kanamycin resistance marker into its coding region, and by in-frame deletion improves nystatin production by 60%, which suggests a negative role of the gene in nystatin biosynthesis.\(^{35}\)

Genes for the biosynthesis of secondary metabolism pathways are commonly grouped together in clusters on

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Table 2. Successful metabolic engineering projects for Streptomyces during the last decade

| Compound     | Application          | Strain         | Genetic/ Metabolic Engineering Approach | Gene disruptions | Gene Transfer Method | Increase | Reference |
|--------------|----------------------|----------------|-----------------------------------------|------------------|----------------------|----------|-----------|
| Actinorhodin | Antibiotic           | *S. coelicolor*| Precursor flux engineering              | λ Red recombinering | Conjugation          | 6-fold   | 32        |
| Clavulanic Acid | Beta-lactamase inhibitor | *S. clavuligerus* | Precursor flux engineering              | Excision and Ligation via restriction enzymes | PEG mediated protoplast transformation | 2-fold   | 31        |
| Clavulanic Acid | Beta-lactamase inhibitor | *S. clavuligerus* | Gene dose increase                      | Excision and Ligation via restriction enzymes | PEG mediated protoplast transformation | 5-fold   | 34        |
| Daptomycin   | Antibiotic           | *S. roseosporus*| GSMM                                    |                  |                      |          | 34.4%     | 46        |
| Doxorubicin  | Cancer chemotherapy  | *S. peucetius*  | Gene upregulation                       | Excision and Ligation via restriction enzymes | PEG mediated protoplast transformation | 4-fold   | 37        |
| ε-polylysine | Food preservative    | Various         | Genome Shuffling                        | UV irradiation    |                      | 3-fold   | 40        |

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the chromosome including their pathway-specific regulatory genes. Pathway-specific regulators can have either positive (activators) or negative (repressors) effects on the expression of gene cluster elements. There are clusters containing different number of pathway-specific positive regulatory genes. The best-known example is \textit{S. coelicolor} that produces several antibiotics (actinorhodin, calcium-dependent antibiotic, undecylprodigiosin, and methylenomycin) and where the onset of their biosynthesis is controlled by specific regulators (\textit{actII-orf4}, \textit{cdaR}, \textit{red}, and \textit{redZ}), while there are several pleiotropic genes (i.e., \textit{afs}, \textit{abs}, and \textit{bld}) affecting antibiotic production and, in addition, the morphological development of the bacteria.\textsuperscript{36} Taking into account all previous observations it seems obvious that deregulation of the expression of secondary metabolite pathways, by overexpression of pathway-specific positive regulators or by inactivation of pathway repressors, is the most intuitive approach for the improvement of their production.

The upregulation of genes is usually controlled by regulatory sequences on the DNA. In \textit{Streptomyces}, there are such regulators that activate the transcription of biosynthetic genes. These are known as SARP (\textit{Streptomyces} antibiotic regulatory proteins). One such as SARP, AfsR-p from \textit{Streptomyces peucetius} have been shown to enhance doxorubicin biosynthesis. In addition, overexpression of AfsR-p in \textit{S. lividans}, \textit{S. clavuligerus}, \textit{S. griseus}, and \textit{S. venezuelae} on a plasmid leads to overproduction of actinorhodin, clavulanic acid, streptomycin, and pikromycin, respectively.\textsuperscript{37} The downregulation of genes is also controlled by regulatory sequences in DNA, however, in this case these would be pathway-specific repressors. The inactivation of these can lead to overproduction of secondary metabolites. This is the case of chromomycin whose production is increased when a pathway-specific transcriptional repressor cmmRII is inactivated in \textit{S. griseus} subsp. \textit{griseus} using a plasmid.\textsuperscript{38} In \textit{S. coelicolor} there is an additional pleiotropic gene, \textit{nudA}, that negatively affects antibiotic production and whose disruption leads to an increase in actinorhodin, calcium-dependent antibiotic, and methylenomycin biosynthesis. In the \textit{nudA} mutant the levels of the pathway-specific regulator \textit{actII-orf4} mRNA are increased.\textsuperscript{39} Genome shuffling involves DNA fragmentation and ligation in order to create possible beneficial mutations. Genome shuffling has been described as a new method that can rapidly enhance secondary metabolite production. The most recent example of this technique is for production of \(\varepsilon\)-PL in five \textit{Streptomyces} species. In this case genome shuffling and interspecific hybridization (combining DNA of two species from the same genus), via UV irradiation allowed for the construction of hybrids, which showed 3-fold improvements in \(\varepsilon\)-PL production.\textsuperscript{40}

\textbf{Genome-Scale Metabolic Models (GSMM)}

In the past decade, despite all the success in secondary metabolite improvement, the approaches of traditional random mutation and selection as well as the rational metabolic engineering, still cannot meet industrial needs. Recently, systems metabolic engineering based on simulation and prediction of mathematic model has provided a reliable method for microbial improvement.\textsuperscript{41} Most recently, metabolic engineering experiments have been performed with the aid of genome-scale metabolic models (GSMM). GSMMs are mathematical models which aim to reconstruct all the metabolic reactions in an organism by
integrated biochemical knowledge and genome annotation. These models can then provide a predictive view of the entire cell which can be useful for downstream applications. Based on this, GSMMs can be used in guiding metabolic engineering of microorganisms for secondary metabolite improvement.

Hemophilus influenza was the first organism for which a GSMM was created and published in 1999. Since then, GSMM has been reconstructed for several bacterial species with improved efficiency and quality. The procedure used to build a GSMM has been described by Durrot et al. and is beyond the scope of this review.

A GSMM was partially constructed for S. roseosporus based on the one already created for S. coelicolor. This model was then used to introduce gene insertions and gene overexpression to create an improved strain in which Daptomycin yield was increased by 34.4%.

Conclusion and Overview

In general, Streptomyces compared with other microbial production hosts, are not a very popular target for metabolic engineering, because they are not as well-characterized as for example E. coli. However, during the last decade, metabolic engineering strategies have increased especially due to the development and application of molecular genetic techniques. This is due to the current growth potential in the bio-based economy, which acts as a strong pulling force for bioprocessing, especially for high-value compounds produced by Streptomyces. Metabolic engineering can be used as a complementary approach to reduce the time needed to optimize a strain.

The number and diversity of secondary metabolites produced by these bacteria is enormous and it has been reported that more than 100,000 different metabolites can be produced, of which only 3% of these have been discovered thus far. Thus, there are still many targets that can be used as targets for metabolic engineering to be discovered to yield new and marketable products.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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