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*Review Article*

# Artificial Chromosomes to Explore and to Exploit Biosynthetic Capabilities of Actinomycetes

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Actinomycetes are an important source of biologically active compounds, like antibiotics, antitumor agents, and immunosuppressors. Genome sequencing is revealing that this class of microorganisms has larger genomes relative to other bacteria and uses a considerable fraction of its coding capacity (5–10%) for the production of mostly cryptic secondary metabolites. To access actinomycetes biosynthetic capabilities or to improve the pharmacokinetic properties and production yields of these chemically complex compounds, genetic manipulation of the producer strains can be performed. Heterologous expression in amenable hosts can be useful to exploit and to explore the genetic potential of actinomycetes and not cultivable but interesting bacteria. Artificial chromosomes that can be stably integrated into the *Streptomyces* genome were constructed and demonstrated to be effective for transferring entire biosynthetic gene clusters from intractable actinomycetes into more suitable hosts. In this paper, the construction of several shuttle *Escherichia coli-Streptomyces* artificial chromosomes is discussed together with old and new strategies applied to improve heterologous production of secondary metabolites.

## 1. Introduction

Actinomycetes, Gram-positive bacteria, represent an important source of biologically active compounds. In fact, they synthesize one-third of the antibiotics commercially available—that is, the macrolide erythromycin, the glycopeptide vancomycin, and the cyclic lipopeptide daptomycin—and other drugs like neuroprotectants (e.g., meridamycin), and anticancer compounds (e.g., migrastatin or geldanamycin) [1]. Most natural products possess complex structures (Figure 1) that are very difficult and expensive to be chemically synthesized and they are obtained at an industrial scale mainly by fermentation processes from the producer microorganism. However, industrial production requires microbial strains that produce unnaturally high yields of a secondary metabolite. In many cases, industrial fermentation yields more than 10 g/L of product; that is, penicillin production process was steadily improved over the years to allow titres of 70 g/L [2]. To increase production yields, classical strain improvement (CSI) or rational genetic methods are used. CSI consists of sequential random mutagenesis and screening of the mutant strains: after each cycle of CSI a population of improved mutants is identified from which the single best performer is selected. CSI does not give information on genes and molecular mechanisms during the improvement and it is likely that some mutations may be detrimental or neutral in term of production.

Rational methods consist of deleting or overexpressing specific genes that, finally, control biosynthesis of a secondary metabolite [3], relying on the knowledge of gene sequences and functions and on suitable protocols for the genetic manipulation of natural producers. The biosynthetic genes devoted to secondary metabolite biosynthesis are organised in clusters that can occasionally reach 100 kb in size or more [4, 5]. Since suitable protocols for the genetic manipulation of natural producers are not always available and given the size of most gene clusters, artificial chromosome-based vectors that can be maintained in *Streptomyces* have been constructed. Such vectors allow genetic information for secondary metabolite production to be transferred from the

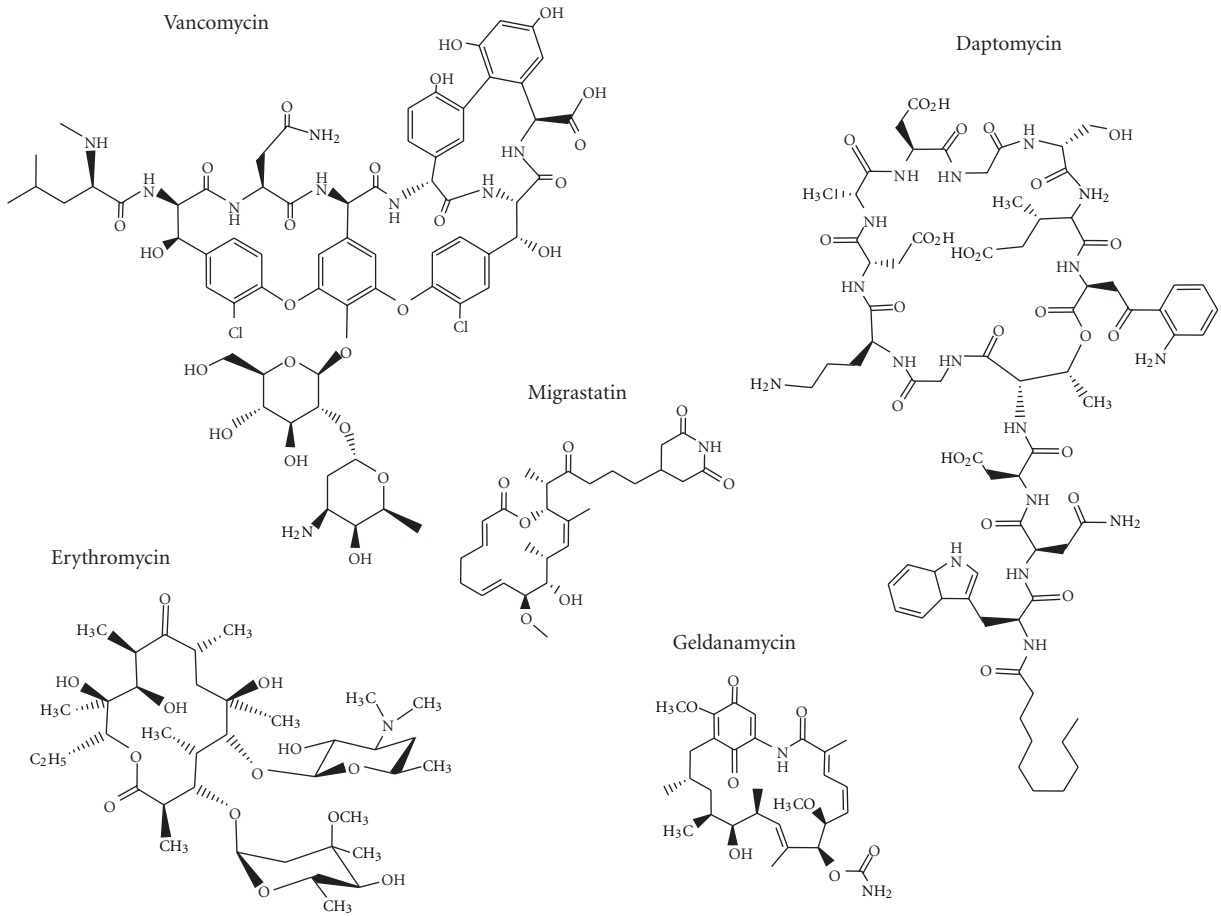


Figure 1: Examples of natural compounds produced by actinomycetes. Vancomycin and daptomycin are synthesized by NRPSs from *Amycolatopsis orientalis* and *Streptomyces roseosporus*, respectively; erythromycin, migrastatin, and meridamycin are produced by PKSs in *Saccharopolyspora erythraea*, *Streptomyces platensis,* and *Streptomyces* sp. *NRRL 30748*.

original producer to a host with well-defined genetics and physiology, where the genes can be expressed. Heterologous expression of large biosynthetic pathways can also be necessary in all cases in which producing bacteria are not cultivable or to examine new metabolites produced by cryptic gene clusters, usually revealed by genome sequencing and mining. This paper deals with artificial chromosome-based vectors and related methods currently available to analyze biosynthetic genes and to express biosynthetic pathways in heterologous systems.

## 2. Handling Large Actinomycete DNA Fragments: Construction of *E. coli-Streptomyces* Artificial Chromosomes

The first step for studying and analyzing biosynthesis at the genetic level is to obtain gene sequences. The recent genomic technologies allow to sequence entire genomes and thereby to identify biosynthetic gene clusters [6]. However, the majority of natural-product-producing microorganisms are refractory to standard gene manipulation techniques, and some are not cultivatable outside their ecological context [7, 8]. For these reasons, the study and modification of whole pathways has greatly benefited from the possibility to manipulate them in vectors, such as cosmids, and artificial chromosomes. Cosmids show high transformation efficiency, with a loading capacity up to 45 kb. Many cosmid libraries of actinomycetes have been successfully constructed to clone and identify biosynthetic gene clusters [9–14]. However, many biosynthetic gene clusters for natural products are larger than the average insert size of common cosmid vectors. To overcome this size limitation, one strategy can be the subcloning of subsets of the biosynthetic gene cluster into compatible expression plasmids followed by their introduction and coexpression in a suitable host strain [15, 16]. Another option is the reassembly of large natural product pathways on a single transferable vector by using the recombineering (recombination-mediated genetic engineering) technology that employs homologous recombination mediated by phage-based recombination systems. Recombineering is independent of the *E. coli* endogenous homologous recombination functions, of restriction sites and of DNA size. Recombineering using the *E. coli* $\lambda$ phage Red pathway, which is mediated by its Exo and Beta proteins, was successfully applied to reconstitute phenalinolactone and

anthramycin gene clusters from two independent cosmids [17, 18] in *E. coli*.

For a single step cloning of inserts larger than 100 kb a more straightforward strategy uses bacterial artificial chromosome (BAC), based on the *E. coli* F factor, and the P1-derived artificial chromosome (PAC), which can carry large DNA fragments (about 100–300 kb) in *E. coli* cells [19]. *E. coli* is not suitable to heterologously express actinomycete genes which are G+C rich [20]. Thus, vectors that can be shuttled between *E. coli*, where library construction and manipulation can be easily conducted, and *Streptomyces*, where actinomycete genes can be expressed (Figure 2), were developed [21–23].

So far, three *E. coli-Streptomyces* artificial chromosomes have been constructed. The features of pPAC-S2 (derived from pCYPAC2 [21]), pSTREPTOBAC V (derived from pBACe3.6 [22]), and pSBAC (derived from pCC1BAC [23]) are briefly reported in Table 1 and maps are given in Figure 3. All of them are replicative in *E. coli* and integrative in *Streptomyces*. Integrative vectors are maintained in the host without selection and are more stable, since metabolic burden and unwanted recombination events are reduced.

Integration into actinomycete chromosome is ensured by the *attP-int* system derived from the temperate phages ΦC31 or ΦBT1, which directs the integration of the vector DNA at the corresponding chromosomal *attB* sites of the host. The availability of two compatible integrating artificial chromosomes can increase the possibility to further manipulate the host. A detrimental effect on endogenous antibiotic production was reported in some producer strains after the integration of vectors in the ΦC31 *attB* site. For example, the integration of nonrecombinant vectors of 11 kb and 48 kb caused a 90 and 59% reduced production of the endogenous glycopeptide A47934 in *Streptomyces toyocaensis* [24–26].

In addition, vectors contain a resistance marker to select recombinant clones. Some antibiotic resistance cassettes, like ampicillin resistance gene, are not useful for selection in *Streptomyces* and different resistance cassettes can be necessary for selection in the two hosts. As an example, the pPAC-S2 contains kanamycin and thiostrepton resistance cassettes used for selecting *E. coli* and *Streptomyces* recombinant clones, respectively. pSTREPTOBAC V and pSBAC contain an apramycin resistance cassette that can be used to select both *E. coli* and *Streptomyces* recombinant clones.

To transfer BACs or PACs from *E. coli* to *Streptomyces* two main strategies can be adopted. In pSTREPTOBAC V and pSBAC, an *oriT* in the vector allows intergeneric conjugation between an *E. coli* strain containing Tra encoding genes (carried in an helper plasmid [7] or into the chromosome [27]) and *Streptomyces*. When *oriT* is not present (i.e., pPAC-S2), a more laborious yet easy and well-established protocol is necessary, which comprises protoplast preparation and PEG-assisted protoplast transformation [7].

## 3. Actinomycete Genomic Libraries Constructed in Artificial Chromosomes

To date, several libraries have been constructed in BAC or PAC artificial chromosomes [22, 23, 28–32] and relevant features of these libraries are mentioned in Table 2.

Using *Sau*3AI, libraries of *S. coelicolor* and *Planobispora rosea* with an average insert size of 60 kb were constructed in pPAC-S2 [28]. A partial genomic library (two-fold coverage) of *S. coelicolor* was first constructed to set up the methodologies, and using the same procedure the first PAC library of an intractable actinomycete, *P. rosea,* with an average insert size of 60 kb and the largest insert of 150 kb was constructed. When the same methodology was applied to *Nonomuraea* sp. ATCC 39727, it was not successful, since its DNA undergoes degradation during PFGE [29, 37]. The PFGE step was therefore omitted, and a high-quality, high molecular weight genomic library of 2,051 recombinant clones with inserts larger than 30 up to 155 kb with an average insert size of 57 kb was constructed in pPAC-S2.

To construct genomic libraries of *S. roseosporus* and *S. platensis, Bam*HI digested genomic DNA was ligated to *Bam*HI digested pSTREPTOBAC V. Libraries of 2000 and 2400 clones with an average insert size of 71 and 75 kb were obtained from *S. roseosporus* and *S. platensis* [22, 31]. The use of the frequently cutting *Sau*3AI allows a more random selection of inserts. *Bam*HI, on the other hand, allows to obtain large inserts due to its less frequent restriction pattern. The *Bam*HI-digested *S. platensis* DNA was fractionated twice by PFGE to reduce the number of small inserts.

Since many genome sequences are nowadays available, more specific approaches can be applied. In the case of *Streptomyces* sp. NRRL 30748, gene sequence analysis had already revealed that two *Mfe*I sites flanked the 90 kb gene cluster devoted to meridamycin biosynthesis and no *Mfe*I sites were present inside the gene cluster. Thus, genomic DNA was digested with *Mfe*I and resulting DNA fragments of approximately 100 kb were ligated to *Eco*RI sites of pSBAC [23].

If the biosynthetic gene cluster was cloned in a BAC/PAC-derived vector that does not contain the integration site for *Streptomyces*, the vector can be provided with an integration cassette and a marker for selection in *Streptomyces*, as in the case of the library of *Streptomyces fradiae* NRRL 18160 constructed in pECBAC. After the identification of a clone containing the gene cluster devoted to A54145 biosynthesis, the clone was modified and used to transform streptomycetes by adding the ΦC31 *attP-int* cassette, the apramycin resistance cassette and *oriT* [30].

## 4. Heterologous Expression of Biosynthetic Gene Clusters from Actinomycetes

Heterologous expression of secondary metabolic gene clusters can allow process development and molecular biological manipulation of the biosynthetic pathways in those cases of native producer strains recalcitrant to manipulation. Even if this strategy appears effective and straightforward, choice of an appropriate host is always challenging.

*E. coli* is not a good host for the expression of actinomycete genes, and heavy manipulation has to be carried out to get biosynthesis of actinomycete products. As an example, to obtain erythromycin biosynthesis, the *E. coli* BL21 (DE3) strain containing the gene for T7 RNA polymerase was

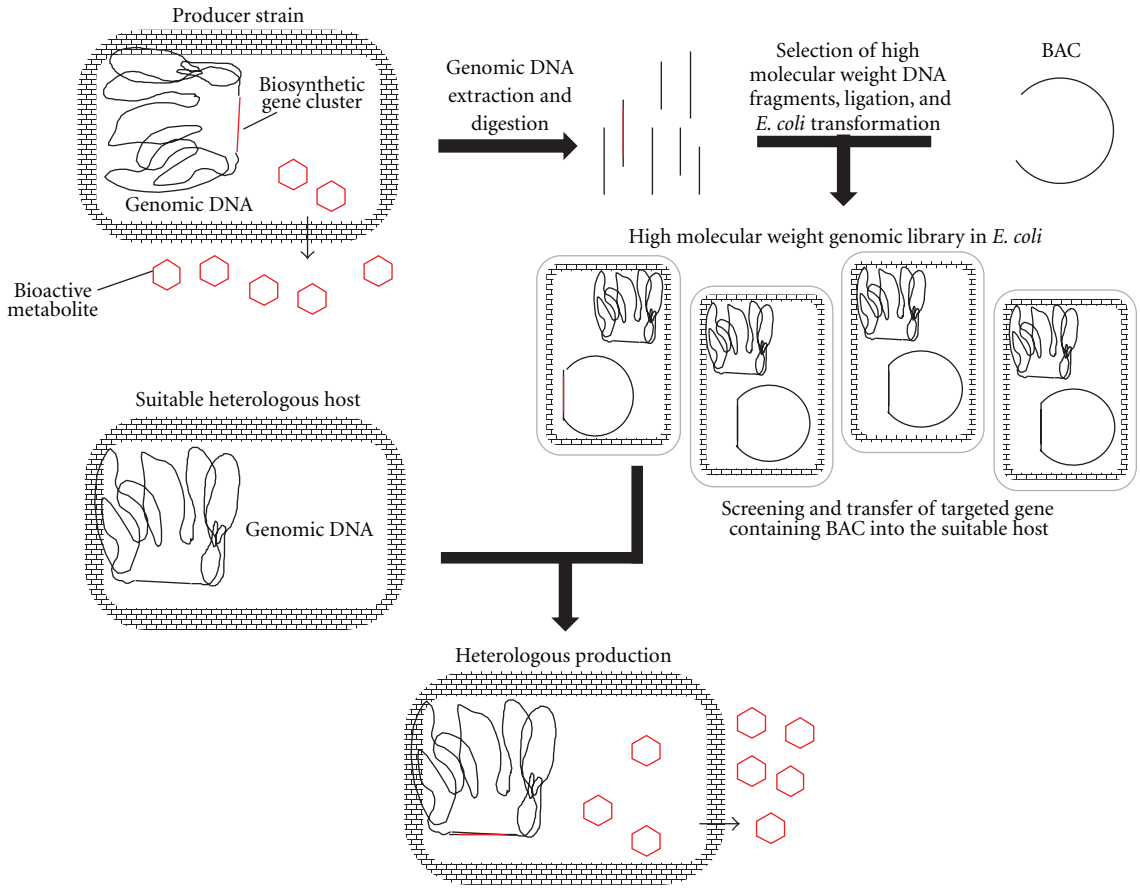


Figure 2: Generalized workflow for heterologous production of natural compounds in an amenable host. A high molecular weight genomic library is constructed and screened to identify one or two clones containing the biosynthetic gene cluster of interest. Once the clone is identified and sequenced to confirm the presence of all the genes, the recombinant vector DNA is used to transform the amenable host. Last, the heterologous production can be improved by changing fermentation process or by genetically manipulating the host.

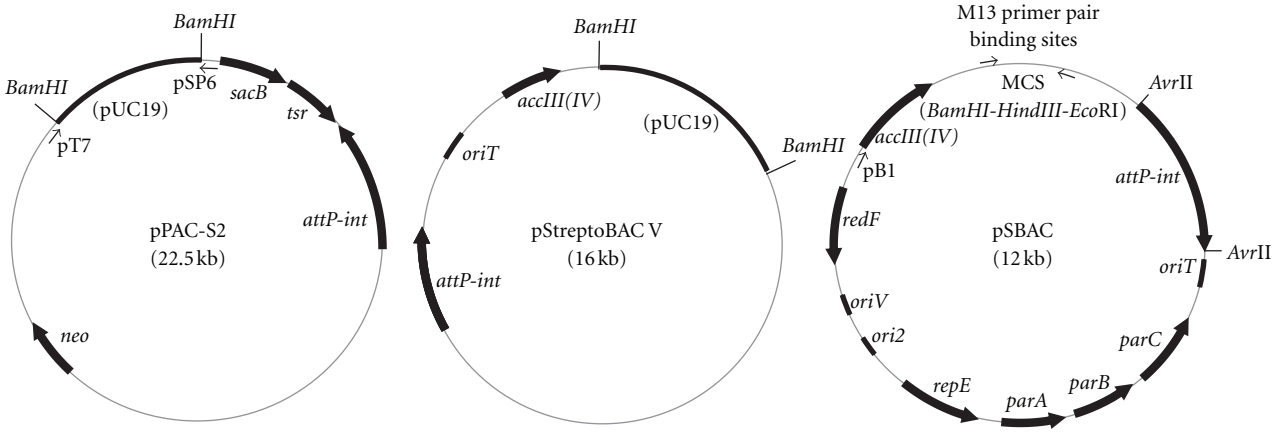


Figure 3: Comparison between physical maps of *E. coli-Streptomyces* artificial chromosomes. The vector pPAC-S2 derives from PAC, while pSTREPTOBAC V and pSBAC from BAC. The main features of the vectors are depicted: the resistance genes (*neo, accIII(IV), tsr* conferring resistance to kanamycin, apramycin, thiostrepton), *attP-int* cassettes for the site-specific integration in *Streptomyces,* restriction sites (*Bam*HI, *Hind*III, *Eco*RI), *sacB* (conferring sensitivity to sucrose when expressed), *oriT* and *oriV*, devoted to intergeneric conjugation and inducible high copy number of the vector, respectively.

Table 1: Shuttle *E. coli-Streptomyces* artificial chromosomes.

| Vector | Derived from | *attP-int* cassette derived from | Resistance to | Reference |
|---|---|---|---|---|
| pPAC-S2 | pCYPAC2 | ΦC31 | Kanamycin/thiostrepton in *E. coli*/*Streptomyces* | [21] |
| pSTREPTOBAC V | pBACe3.6 | ΦC31 | Apramycin in *E. coli*/*Streptomyces* | [22] |
| pSBAC | pCC1BAC | *φ*BT1 | Apramycin in *E. coli*/*Streptomyces* | [23] |

Table 2: Actinomycetes genomic libraries constructed in artificial chromosomes.

| Source | Genome coverage | Average insert size | Genomic DNA digested with | Vector | Reference |
|---|---|---|---|---|---|
| *Planobispora rosea ATCC 53773* | 5X | 60 kb | *Sau3*AI | pPAC-S2 | [28] |
| *Nonomuraea sp. ATCC 3972* | 5X | 57 kb | *Sau3*AI | pPAC-S2 | [29] |
| *S. roseosporus NRRL 11379* | 17X | 71 kb | *Bam*HI | pSTREPTOBAC V | [22] |
| *S. platensis NRRL18993* | 22X | 75 kb | *Bam*HI | pSTREPTOBAC V | [31] |
| *Streptomyces sp. NRRL 30748* | NR | NR | *Mfe*I | pSBAC* | [23] |
| *S. autolyticus JX-47* | 50X | 150 kb | *Sau3*AI | pIndigoBAC5* | [32] |

NR: not reported.
*Not integrative in *Streptomyces*.

transformed with four plasmids: (i) pHZT1 and (ii) pHZT2 containing all *ery* genes organized in two operons under T7 promoter control; (iii) pHZT4 containing *eryK*, the last gene of one of the operons, since it was not expressed at high level; and (iv) plasmid pGro7 containing the genes for the *E. coli* GroEL/ES chaperone system to aid protein folding and/or association of actinomycete gene products. Only the strain carrying the four plasmids produced erythromycin A, while the same *E. coli* strains containing only pHZT1 and pHZT2 did not. This very labour-intensive, time-consuming procedure was specific for erythromycin [38], and a similar strategy could not always be applied for the synthesis of other antibiotic compounds that may require "specific" precursors that *E. coli* cannot provide.

Streptomycetes are naturally the preferred hosts of actinomycete genes. Compared to other actinomycetes, *Streptomyces* is more amenable for strain improvement, grows more rapidly, and generally has the biosynthetic apparatus and primary precursors necessary to support natural product synthesis from exogenous pathways. Among streptomycetes, *S. coelicolor* and *S. lividans* have been widely used as heterologous hosts.

*S. coelicolor* is considered the model species among actinomycetes and a large array of genetic tools, including its genome sequence, is available to understand and manipulate the organism [7, 39]. Although there are many advantages of using *S. coelicolor* as a host, plasmid-free derivatives of *S. lividans*, a close relative of *S. coelicolor*, have often been used for heterologous production (Table 3) [40, 41]. The primary reason is the absence of a strong restriction system, like in *S. coelicolor*, which heavily restricts methylated DNA [42].

Other streptomycetes have already been used for heterologous expression of secondary metabolite gene clusters, like *Streptomyces albus* G J1074 [35], and other industrial strains, such as *S. avermitilis*. *S. albus* G J1074 is a mutant defective in *Sal*I restriction and modification, readily transformable cloning host that has been used for the expression of several other secondary metabolite gene clusters [35]. The industrial microorganism *S. avermitilis*, producer of the anthelmintic macrolide avermectins, has been already optimized for the efficient supply of primary metabolic precursors and biochemical energy to support multistep biosynthesis [43, 44].

*4.1. Optimization of Heterologous Production in Streptomycetes.* In some cases heterologous production is either absent or so low that manipulation of the host is necessary. Different strategies, both genetics- and physiology-driven, can be explored in a well-defined host. Fermentation-based approaches can consist of varying carbon or nitrogen sources or different phosphate levels in the medium and in feeding with a biosynthetic precursor that may be limiting during the growth phase. Genetic methods can target both host and cloned genes. Genomic analysis of streptomycetes has revealed that these microorganisms have large linear chromosomes that harbour over 20 gene clusters for secondary metabolites that could compete for precursors or could interfere with chemical analysis. Thus, the deletion of host gene clusters devoted to biosynthesis of other endogenous metabolites can increase precursor supply and, thus, improve heterologous production [33, 34, 36, 45, 46]; in addition, there are point mutations in *rpoB* and *rpsL* genes, coding for RNA polymerase beta-subunit and 30S ribosomal subunit protein S12, respectively, known for increasing antibiotic production [45, 47]. Although the underlying molecular mechanism is not understood, these mutations have a pleiotropic effect on levels of secondary metabolite production that is achieved, at least in part, by elevated transcript levels.

*S. coelicolor* produces at least four metabolites with antibiotic activity: the Type II polyketide actinorhodin (Act), the NPRS/PKS-derived prodiginines (Red), the NRPS-derived calcium-dependent antibiotic (CDA), and the TypeI polyketide yellow pigment CPK [39]. In the last two years, two groups put their efforts in the engineering of *S. coelicolor*

Table 3: Streptomycetes hosts.

| Strain | Genotype | Reference |
|---|---|---|
| *S. lividans* TK23 | *spc* $^{-}$ SLP2$^{-}$ SLP3$^{-}$ | [7] |
| *S. lividans* TK24 | *str6* $^{-}$ SLP2$^{-}$ SLP3$^{-}$ | [7] |
| *S. lividans* TK64 | *pro-2 str-6* SLP2$^{-}$ SLP3$^{-}$ | [7] |
| *S. lividans* K4-114 | *pro-2 str-6SLP2*$^{-}$ *SLP3*$^{-}$Δ*act::ermE Streptomyces* | [33] |
| *S. coelicolor* M512 | Δ*redD* Δ actII-ORF4 SCP1$^{-}$ SCP2$^{-}$ | [34] |
| *S. albus G J1074* | *ilv-1 sal-2* | [35] |
| *S. avermitilis* SUKA4/SUKA5 | Δ(*gap1-ptlL*)::*ermE* (Δ3,745,502–3,758,936 nt) | [36] |

in order to construct suitable production hosts [45, 46]. In one case four of these endogenous secondary metabolite gene clusters were deleted and point mutations in *rpoB* and *rpsL* genes were introduced [45]. In the more recent case, sequential deletions of all ten polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) biosynthetic gene clusters and a 900-kb subtelomeric sequence (total ca. 1.22 Mb, 14% of the genome) from *S. coelicolor* chromosome were performed to construct derivative strains [46]. In addition to lacking antibacterial activity, the engineered strains possess relatively simple extracellular metabolite profiles and they could markedly facilitate the discovery of new compounds by heterologous expression of cloned gene clusters, particularly the numerous cryptic secondary metabolic gene clusters that are prevalent within actinomycete genome sequences [45, 46].

*S. lividans* has been largely used as heterologous host for its more relaxed restriction/modification system [42], but some strains of *S. lividans* under many fermentation conditions produce actinorhodin that interferes with the detection and purification of other secondary metabolites, so part of the *act* gene cluster was deleted in K4-114, therefore providing a cleaner background for heterologous expression [33].

The industrial microorganism *S. avermitilis* was manipulated by deletion of the telomeric ends of the linear chromosome, which contain nonvital genes, often antibiotic biosynthesis-related [36]. A region of more than 1.4 Mb was deleted stepwise from the 9.02 Mb *S. avermitilis* linear chromosome to generate a series of defined deletion mutants (i.e., SUKA4, SUKA5), that did not produce any of the major endogenous secondary metabolites found in the parent strain.

Also foreign genes can be manipulated, that is, strong promoters can be added to drive transcription of genes or operons, otherwise weakly transcribed.

Here we discuss the successful production of daptomycin (from *dpt* genes), meridamycin (from *mer* genes), and migrastatin (from *mgs* genes), pointing out the strategies applied (Figure 4). Firstly, artificial chromosome libraries from *S. roseosporus* NRRL 11379, *S. platensis* NRRL18993, and *Streptomyces* sp. NRRL 30748 were screened by PCR or hybridization for the presence of *dpt, mgs, mer,* and *lpt* gene clusters, respectively. Once sequenced, the relevant clones were used to transform various *Streptomyces* hosts, where they were stably integrated and maintained.

*4.1.1. Production of Daptomycin in S. lividans.* The daptomycin gene cluster was transferred in *S. lividans* TK64 and TK23. In both cases, the recombinant strains produced the lipopeptide with a low titre and a high amount of actinorhodin [22, 41]. To reduce the background effect of native metabolites, the *S. lividans* TK23 host was manipulated by inactivating actinorhodin production through replacement of a portion of the gene cluster with a resistance gene, but this did not result in a yield improvement. An improvement in daptomycin biosynthesis was obtained by increasing the levels of $K_2HPO_4$ during fermentation. It is likely that phosphate, often reported as an inhibitor of antibiotic production, inhibited host metabolite biosynthesis pathways, whose precursors could be necessary for daptomycin biosynthesis. Adjusting the level of phosphate in the medium the yield of the lipopeptide was increased from 20 to 55 mg/L, corresponding to one-third of the yield of the natural producer [22, 41].

*4.1.2. Production of Meridamycin in S. lividans.* In the case of meridamycin biosynthesis, the transfer of the 90 kb gene cluster to *S. lividans* TK24 and K4-114 did not lead to the detection of any heterologous metabolites, since PKS genes were not properly transcribed, possibly due to the size of operon (3 genes for a 78 kb operon). Indeed, the cloning of a strong promoter (*ermE* promoter) upstream of the *mer* genes coding for the PKS allowed the detection of approximately 100 $\mu$g/L of meridamycin in the fermentation extract. To further increase the yield, a fermentation strategy based on feeding with biosynthetic precursors was applied. The supply of ethylmalonyl-CoA, used as an extender unit in meridamycin biosynthesis, is a critical factor that limits the synthesis of some polyketides by heterologous hosts. In fact, when *S. lividans* containing *mer* gene cluster with PKS genes under *ermE* control, was supplemented with diethyl malonate, which is an effective precursor for ethylmalonyl-CoA, the production increased about 2-fold (240 $\mu$g/L). On the other hand, feeding with pipecolic acid, another unique precursor for the biosynthesis of meridamycin, did not increase production [23].

*4.1.3. Production of Migrastatin in Streptomycetes.* To find the most suitable host to express the biosynthetic gene cluster for the production of the polyketide migrastatin of *S. platensis* NRRL18993, five *Streptomyces* hosts were selected: *S. lividans* K4-114, *S. coelicolor* M512, *S. albus G J1074*, and the two

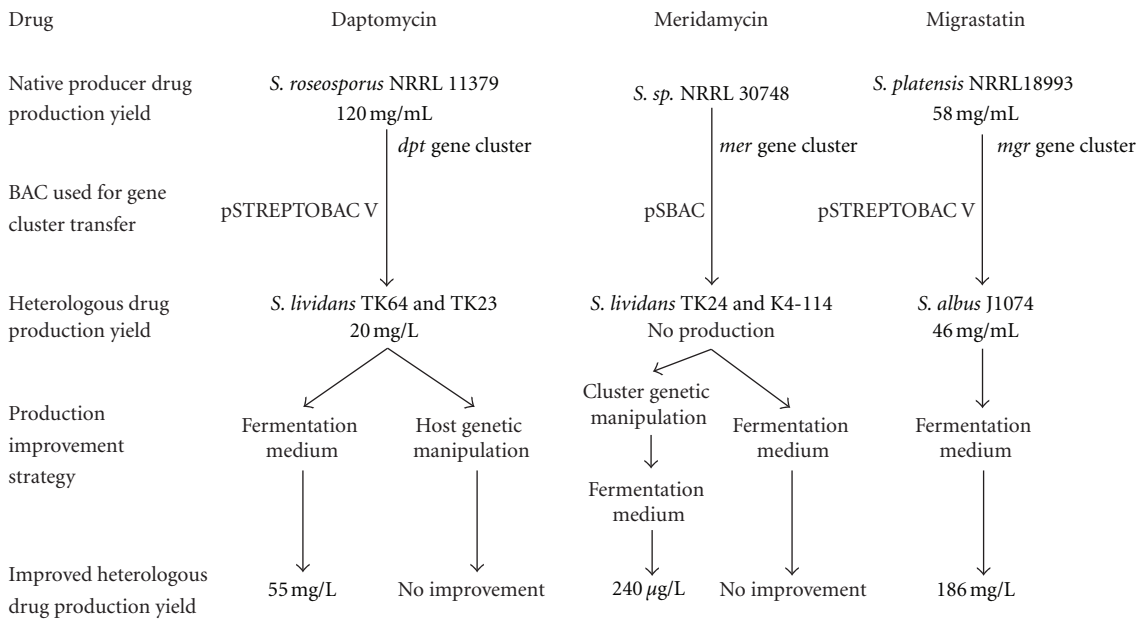


Figure 4: Strategies adopted to improve heterologous production of daptomycin, meridamycin, and migrastatin in heterologous hosts. Under "fermentation strategies" fall feeding with biosynthetic precursors or growth in presence of specific nutrients. Host genetic manipulation indicates the inactivation of host biosynthetic pathways or mutations in genes (*rpsL* and *rpoB*) known for increasing secondary metabolite production. Genetic cluster manipulation refers to the cloning of a strong promoter upstream of foreign genes which are weakly transcribed. Details are provided in the text.

*S. avermitilis strains*, SUKA4 and SUKA5. All the five strains produced the metabolite, but the titres, in two media already optimized for the natural producer, were similar to or less than those obtained with *S. platensis*. The best producers were *S. albus* that produced approximately 46 mg/L in B2 medium (natural producer 58 mg/L), and *S. lividans* K4-114 that produced 15 mg/L (control 17 mg/L) in R2YED medium. Adjusting fermentation conditions, an improvement of the titer (appr. 186 mg/L) was registered in *S. albus*. In addition, feeding with $NaHCO_3$ that plays a critical role in the biosynthesis of malonyl-CoA, the major precursor for migrastatin biosynthesis, allowed *S. albus* to produce migrastatin at a titer of 213.8 mg/L, about 5-fold higher than the originally [31, 48].

*4.1.4. Production of the Lipopeptide A54145 in S. ambofaciens and S. roseosporus.* It is noteworthy that also the production of the lipopeptide A54145 from *S. fradiae* was obtained in streptomycetes hosts, but the vector, pECBAC1, was not an *E. coli-Streptomyces* shuttle vector. The recombinant vector, containing the entire *lpt* gene cluster was modified by adding the ΦC31 *attP-int* integration cassette after library construction [30]. The *lpt* gene cluster of *S. fradiae* was transferred into *S. ambofaciens* and *S. roseosporus* strains genetically manipulated in a such way to not being anymore able to produce spiramycin [49] and daptomycin [50], respectively. Interestingly, both sets of heterologous strains were more efficient than wild type *S. fradiae* at producing the lipopeptide, but less productive than the *S. fradiae* high-producer control.

## 5. Future Perspectives

Artificial chromosomes are useful tools to exploit or to explore the genetic potential of intractable actinomycetes, by creating, for example, large-insert libraries from strains of interest, in order to isolate one or more large gene clusters [22, 23, 28–32], or by reconstructing gene clusters that are already available as a set of smaller fragments cloned in lower capacity vectors [17, 18, 51, 52]. To be transferred in amenable hosts, BAC and PAC are provided with means to be maintained in manipulable streptomycetes, which can synthesize foreign complex molecules, such as antibiotics [21–23]. Sometimes, heterologous expression of actinomycetes biosynthetic gene clusters was successful after changing fermentation conditions, that is, feeding with a biosynthetic precursor, minimizing background endogenous activities or after cloning strong promoters upstream of production genes weakly transcribed. The examples here described can be considered as promising starting points to successfully express heterologously valuable biosynthetic products. A significant improvement in heterologous production will surely come from new technologies, like next generation sequencing and gene stitching and synthesis. Indeed, the genome sequences of both the producing strains and of the hosts can help to find the minimal set of genes necessary to maintain heterologous production in a clean background and to avoid interference between foreign gene products and host that in some cases were reported [53, 54].

A possibility could be to create artificial hosts that maintain as less endogenous genes as possible and that contain the specific genes to provide primary metabolites and all the cofactors necessary to synthesize a specific secondary

metabolite. Some studies in this direction have just started with the deletion of large DNA fragments from *S. avermitilis* and *S. coelicolor* chromosomes [36, 46]. More efforts are needed to obtain a universal suitable host of actinomycete gene clusters; although *S. avermitilis* and *S. coelicolor* strains with minimized genomes have been constructed and shown to produce more metabolites than natural producers, further manipulation of their genomes followed by the improvement of the yields will hopefully occur with the help of specific experiments of gene stitching and synthesis. The genome sequencing of *Streptomyces* species (e.g., *S. coelicolor* and *S. avermitilis* [39, 43]) has remarkably revealed that each strain has the genetic information to produce a large number (e.g., 23 and 32, resp.) of secondary metabolites. This implies that as much as 90% of the chemical potential of these organisms remains undiscovered by the conventional screening programs. Genome mining offers a powerful method for tapping into these cryptic natural products [55]. For discovery of new compounds by genome mining, heterologous expression could be employed to examine new products by these new gene clusters.

Heterologous expression through artificial chromosomes may provide the possibility to access gene clusters for the production of bioactive metabolites from the large number of uncultured bacteria present in the environment without the need to actually isolate them [56–61]. Growing many of these organisms at large scale for production purposes is unlikely to be practical but their biosynthetic gene clusters are particularly attractive targets for heterologous expression in a genetically amenable host.

For all these cases, it will be important to develop a suite of streptomycete hosts for high-level heterologous expression of specific types of secondary metabolites, and to facilitate rapid identification and testing of novel products. Given the relatively recent success of heterologous complex natural product biosynthesis, these tools are just beginning to be applied, but they appear to be very promising. Such approaches, in addition to omics global characterization techniques and more traditional process engineering steps, will be of paramount importance for the maximization of natural product outputs from heterologous hosts.

When the new technologies (e.g., gene synthesis) will be cheap and there will be no more novel activities and genes to discover, it is likely that cloning strategy will be abandoned, but at the moment this perspective is quite far.

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