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REVIEW ARTICLE

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Heterologous expression of natural product biosynthetic gene clusters in *Streptomyces coelicolor*: from genome mining to manipulation of biosynthetic pathways

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Abstract Heterologous gene expression is one of the main strategies used to access the full biosynthetic potential of actinomycetes, as well as to study the metabolic pathways of natural product biosynthesis and to create unnatural pathways. *Streptomyces coelicolor* A3(2) is the most studied member of the actinomycetes, bacteria renowned for their prolific capacity to synthesize a wide range of biologically active specialized metabolites. We review here the use of strains of this species for the heterologous production of structurally diverse actinomycete natural products.

Keywords Actinomycete · Heterologous host · Secondary metabolite · Natural product biosynthesis

Introduction

Natural products, also referred to as "secondary metabolites" or "specialized metabolites", account for between one and two-thirds of all therapeutic compounds (depending on the data source) used either directly as isolated from nature or as semi-synthetic derivatives; about 35 % of these are of microbial origin. Strikingly, nearly 70 % of anti-infectives used in medicine are natural products or their derivatives [1, 2]. Although for the past couple of decades the pharmaceutical industry has focused largely on synthetic chemical libraries as a source of new drug leads, there is now renewed interest in natural products [3–5]. This is partly because sequencing of the genomes of producing micro-organisms has revealed a much larger capacity for the biosynthesis of specialized metabolites than previously thought, raising the prospect of finding new structural classes of natural products with useful biological activities.

The actinomycetes, high G+C Gram-positive bacteria of terrestrial and marine origin, produce more than 40 % of all known bioactive natural products of microbial origin [1]. Moreover, 35 % of all marketed antibiotic formulations contain an active ingredient derived from an actinomycete; since most antibiotics are semisynthetic derivatives of a few natural products, actinomycetes produce an impressive 76 % of all original natural product scaffolds used as anti-infective agents [6].

However, much of the biosynthetic potential of these organisms is not observed under laboratory conditions. Bioinformatic analysis of the genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2) first revealed the presence of multiple cryptic gene clusters, i.e., clusters of genes without a known metabolic product [7, 8]. Such cryptic gene clusters may or may not be transcribed under laboratory conditions. Since then, the affordability of high-throughput DNA sequencing has facilitated the analysis of the genome sequences of an increasing number of actinomycetes, revealing that cryptic gene clusters are a general feature of these organisms. This has led to the development of "genome mining" as a new strategy for natural product discovery [9, 10] to which this issue of *JIMB* is mostly devoted.

"Genome mining" can be defined as the use of bioinformatics, molecular genetics, and natural product analytical chemistry to access the metabolic product of a gene cluster found in the genome of an organism. There are two main approaches to genome mining: (1) to activate the expression of a transcriptionally silent cryptic gene cluster by genetic manipulation of the producing organism, e.g.,

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by deleting or over-expressing putative negative or positive transcriptional regulators, respectively [11, 12]; reviewed in this issue: [13, 14]; (2) to clone a fragment of the genome of the producing organism containing the gene cluster and express it in a suitable heterologous host.

Heterologous expression can be used for a number of purposes. For example: to demonstrate that the complete set of genes required for the biosynthesis of a particular metabolite has been cloned; to obtain the metabolic product of a cryptic gene cluster from an organism that is difficult to culture or that is not genetically amenable; and to obtain unnatural metabolites (by combining genes from different biosynthetic pathways or by expressing mutated gene clusters). General approaches and methods have been discussed elsewhere [15].

Streptomyces coelicolor as a heterologous host

The production of a typical natural product relies on numerous cellular processes, all of which are highly dependent on the genetics and biochemistry of the producing organism. Thus, for effective heterologous expression, the use of a host species as closely related as possible to the organism from which the genes to be expressed were isolated will likely prove optimal. The use of *Streptomyces* (order Actinomycetales/suborder Streptomycineae) and *Saccharopolyspora* (order Actinomycetales/suborder Pseudonocardineae) strains as heterologous hosts have been reviewed recently [16].

S. coelicolor is genetically the most studied actinomycete species. Its chromosome was fully sequenced and annotated over a decade ago [7] and a large array of genetic tools are available to manipulate the organism [17, 18]. These include promoters for gene expression (e.g., the *ermE*p*, *tipA*p, and *tcp*830p promoters for constitutive, inducible and derepressible gene transcription, respectively) and the ability to manipulate and integrate large-insert genomic libraries into the host's chromosome (e.g., Ref. [19]; for an overview see Ref. [15]). Furthermore, there is considerable understanding of the regulatory mechanisms that control the processes of both physiological and frequently linked morphological differentiation in this species [20].

Like many if not most streptomycetes, *S. coelicolor* produces both polyketides and non-ribosomal peptides, and thus possesses the necessary primary metabolism to supply the precursors for both of these important classes of compounds. Most of the specialized metabolites produced by this species have now been identified and structurally characterized [21–35]. Moreover, methods for affordable small-scale cultivation of *S. coelicolor* have been developed, allowing rapid screening at reasonable throughput for laboratories without specialized facilities for high-throughput screening [36, 37].

Unsurprisingly then, *S. coelicolor* has been used by many research groups as a host for the heterologous expression of biosynthetic gene clusters isolated from other actinomycetes.

Strains of *Streptomyces coelicolor* used as heterologous hosts

One of the desirable characteristics of a host strain is limited production of its own specialized metabolites so that metabolic precursors and energy can be directed into the synthesis of the heterologous product. Another is the lack of production of compounds with antibiotic activity that could interfere with activity-based screens for the products of cloned gene clusters. Five *S. coelicolor* strains that largely fulfill these requirements, and that lack both of the endogenous plasmids SCP1 and SCP2, have been used as heterologous hosts:

S. coelicolor CH999 [38]: constructed with the main aim of producing polyketides by deleting the actinorhodin gene cluster and incorporating mutations in the gene cluster for the prodiginines that abolish their production.
S. coelicolor M512 [39]: constructed to study the regulation of actinorhodin and prodiginine production by the pleiotropic regulatory protein AfsR. Production of both antibiotics was abolished by marker-less deletion of the pathway transcriptional activator genes *actII*-ORFIV and *redD*, respectively. This strain has proved popular because it does not carry any foreign DNA, including antibiotic resistance genes.
S. coelicolor M1146 [40]: constructed by sequential marker-less deletion of most of the gene clusters for the production of actinorhodin (*act*), prodiginines (*red*), coelimycin (*cpk*), and the calcium-dependent antibiotic (*cda*). This strain was specifically designed for use as a heterologous expression host, and the deletion of the four gene clusters markedly reduces the possibility of enzymatic interference by host enzymes with an introduced biosynthetic pathway. M1146 also possesses a much simplified extracellular metabolic profile, markedly facilitating the identification of heterologously produced metabolites [15].
S. coelicolor M1152 and M1154 [40]: obtained from M1146 after the introduction of "Ochi-type" mutations—either a single *rpoB* point mutation (M1152) or double *rpoB* and *rpsL* point mutations (M1154)—providing higher levels of production of specialized metabolites due largely to increased levels of gene transcription (reviewed by Ochi and Hosaka [41]). These strains are being adopted widely by the research community (see Table 1). Production of heterologous metabolites is usually much higher than in M1146 [19, 40] or other streptomycete hosts [42].

Table 1 Gene clusters expressed in the optimized *S. coelicolor* hosts strains M1146, M1152, and M1154

Metabolite	Metabolite class	Natural producing micro-organism	Aims	Yield	References
Cypemycin	Linaridin (RiPP)	*Streptomyces* sp. OH-4156	2, 3	Not reported	[65]
Grisemycin	Linaridin (RiPP)	*Streptomyces griseus* IFO 13350	2, 4	Not reported	[66]
Actagardine	Lantibiotic (RiPP)	*Actinoplanes garbadinensis* ATCC31049	2, 3	Not reported	[56]
Planosporicin	Lantibiotic (RiPP)	*Planomonospora alba*	2	Not reported	[62]
GE37468	Thiopeptide (RiPP)	*Streptomyces* sp. ATCC 55365	6	Not reported	[42]
Napsamycin	Uridylpeptide	*Streptomyces* sp. DSM5940	2	Not reported	[67]
Clorobiocin	Aminocoumarin	*Streptomyces roseochromogenes* var. *oscitans* DS 12.976	5	158 mg/l	[36]
Coumermycin A1	Aminocoumarin	*Streptomyces rishiriensis* DSM 40489	5	160 mg/l	[36]
Caprazamycin	Liponucleoside	*Streptomyces* sp. MK730-62F2	5	152 mg/l	[36]
FK506/FK520 (tacrolimus)	Polyketide	*Streptomyces tsukubaensis* NRRL 18488	2	2.81 mg/l	[19]
Merochlorins	Polyketide-terpenoid	*Streptomyces* sp. CNH-189	2, 3	Not reported	[47]
Gougerotin	Peptidyl nucleoside	*Streptomyces graminearus* CGMCC 4.506	2, 3	Not reported	[68]
Endophenazine	Phenazine	*Streptomyces anulatus* 9663	2, 3	20 mg/l	[52]
Roseoflavin	Riboflavin (vitamin B2)	*Streptomyces davawensis* JCM 4913	2	0.4 mg/l	[69]
Staurosporine and streptocarbazoles	Indolocarbazoles	*Streptomyces sanyensis* FMA	2	Not reported	[46]
Holomycin	Thiopeptide (NRPS)	*Streptomyces clavuligerus* ATCC27064	2, 3	Not reported	[70]
Chloramphenicol	Shikimic acid pathway	*Streptomyces venezuelae*	1	40 mg/l	[40]
Congocidine	Pyrrole-amide (oligopyrrole)	*Streptomyces ambofaciens*	1	Not reported	[40]
Tunicamycin	Fatty-acyl nucleoside	*Streptomyces chartreusis* NRL3882	2, 3	Not reported	[43, 44]
Unsuccessful product detection					
Microbisporicin	Lantibiotic	*Microbispora coralina*	2	0	[60]
Platencin	Platencin and platensimycin	*Streptomyces platensis* MA7327	2, 3	0	[54]

1, validation of host strains; 2, cloning and characterization of biosynthetic gene cluster; 3, study of biosynthetic pathway; 4, characterization of metabolite; 5, optimization of production; 6, generation of unnatural compounds; *RiPP*, ribosomally synthesized post-translationally modified peptide

Classes of secondary metabolites successfully produced

S. coelicolor is proving to be a suitable heterologous host for an increasing number of structural classes of secondary metabolites. In addition to the well-known polyketides and non-ribosomal peptides, metabolites derived from other complex biosynthetic pathways have been produced by heterologous expression of the corresponding gene cluster in this species. Examples of gene clusters expressed in the engineered *S. coelicolor* strains are summarized in Table 1. These include gene clusters for lantibiotics (e.g., actagardine and planosporicin) originating from actinomycetes phylogenetically distant from *Streptomyces*.

Characterization of biosynthetic pathways

An important advantage of using such well characterized strains as hosts is that their genetics and metabolism are reasonably well understood, allowing the identification and study of metabolic intermediates of the heterologous pathway that are frequently produced and accumulated at low levels. As an example, the biosynthesis of the nucleoside antibiotic tunicamycin had been proposed to occur via an unusual 4-keto-5,6-ene-*N*-acetyl-sugar intermediate [43]; in vitro studies with purified enzymes identified UDP-6-deoxy-5,6-ene-*N*-acetyl-galactose as the most likely candidate. To demonstrate the involvement of this sugar in vivo, a copy of the tunicamycin gene cluster that had been mutated in a gene required for its subsequent processing was expressed in *S. coelicolor* M1152, leading to the accumulation of the predicted intermediate and its detection by LC–MS analysis [44].

Heterologous expression can also be very useful when the production of a metabolite by the natural producer is insufficient to allow investigation of the biosynthetic pathway. For example, after attempts to improve erythreapeptin production in the natural producer *Saccharopolyspora erythraea* failed to yield sufficient material for structural characterization, Süssmuth and coworkers [45] achieved their goals by expressing the biosynthetic gene

cluster for the type-III lantibiotic in *S. coelicolor* M1146 and *S. lividans* TK24.

Actinobacteria isolated from marine environments are becoming a promising new source of natural products, and several examples of gene clusters isolated from marine actinomycetes have been published recently (e.g., Ref. [46]). A particularly interesting example of heterologous expression of such a gene cluster in *S. coelicolor* is the study of novel enzymatic reactions carried out by rare haloperoxidases during the biosynthesis of merochlorins, whose gene cluster was isolated from a marine *Streptomyces* species [47].

In another interesting example, Müller and coworkers identified the gene cluster for bottromycin biosynthesis by genome mining of *Streptomyces* sp. BC16019, but had to rely on heterologous expression in *S. coelicolor* to study the function of several of the biosynthetic genes. Deletion of these genes in the natural producer could not be obtained by standardly used double cross-over homologous recombination [48].

Creation of unnatural pathways

The generation of novel chemical structures by combining genes from different pathways or by abolishing the synthesis of a precursor and feeding unnatural precursors (mutasynthesis) are technologies known since the mid 1980s [49]. However, it is with the current availability and affordability of gene synthesis that its full potential is starting to be realized.

For example, Young and coworkers [42] generated new variants of the ribosomally synthesized peptide antibiotic GE37468. They expressed mutated precursor peptide genes, in which up to seven codons had been randomized, in *S. coelicolor* M1152 carrying the genes for GE37468 post-translational modification and secretion. They generated a library of 29 novel unnatural variants of the antibiotic that were subsequently assayed for bioactivity.

A targeted approach was chosen by Alt and coworkers [50] to incorporate specific structural motifs into the aminocoumarin antibiotic clorobiocin. They aimed to replace the 3-dimethylallyl-4-hydroxybenzoyl moiety of this compound with catechol, thus mimicking a siderophore that could be actively taken up by Gram-negative bacteria (clorobiocin is active only against Gram-positive bacteria). Having failed with mutasynthesis (annulling a key gene in the biosynthesis of the hydroxybenzoyl ring and feeding analogs to the resulting mutant), the authors designed an unnatural biosynthetic pathway for 3,4-dihydroxy benzoic acid (DHBA) by synthesizing genes with a codon usage optimized for *Streptomyces* [51]. The pathway included chorismate pyruvate lyase (UbiC) from *Escherichia coli* and 4-hydroxy benzoate-3-hydroxylase (PobA) from *Corynebacterium cyclohexanicum*; the genes were translationally coupled and placed under the control of the strong constitutive promoter *ermE*p*. Expression of these genes in *S. coelicolor* M512 led to the production of DHBA and its subsequent incorporation into the unnatural and heterologously produced aminocoumarin derivative containing the catechol moiety.

Limitations and challenges

S. coelicolor M1152 and M1154 have proved useful for the heterologous production of many different natural products, often proving superior to other *Streptomyces* strains and species [42]. But there are also examples of failed expression in *S. coelicolor*. This seems to be highly dependent on the gene cluster, and it is not always readily explained. An interesting example is the study of the endophenazine gene cluster from *Streptomyces anulatus* [52]; *S. coelicolor* M512 produced much larger amounts of the expected endophenazine A than M1146 or M1154, although the authors found that these two strains accumulated large amounts of a glutamine adduct that they called endophenazine E, also detectable at much lower levels in M512. In a subsequent paper with a similar gene cluster, the same authors speculate that the glutamination could be a defense mechanism of *S. coelicolor*, since the glutamine adduct does not have antibiotic activity [53].

Another intriguing example is the heterologous expression of platencin biosynthetic gene cluster from *Streptomyces platensis* MA7327. Smanski and coworkers [54] were able to detect production of platencin only in *S. lividans* (strain K4-114), while three different *S. coelicolor* strains (CH999, M1146 and M1154) and *Streptomyces albus* J1074 failed to produce any detectable compound. Production in *S. lividans* was detected only after deletion of the pathway specific regulator *ptnR1*, i.e., it was necessary to remove the negative transcriptional regulation to achieve the heterologous expression. Why this did not work in the other *Streptomyces* strains is not clear.

In other cases, levels of production were markedly increased after exchanging the native transcriptional promoters of the gene cluster of interest by presumably stronger constitutive promoters. Du and coworkers [55] obtained a tenfold increase in gougerotin production after replacing the native promoters with that of *hrdB* (which encodes the major sigma factor of *S. coelicolor*), reaching 0.5 mg/l of production in *S. coelicolor* M1146.

Another limitation may be the sensitivity of the host strain to the metabolite being produced. In the aforementioned case of bottromycin biosynthesis in *S. coelicolor*, heterologous production of the antibiotic was very low until the authors used the strong constitutive promoter

*ermE*p* to drive transcription of the gene encoding the putative bottromycin transporter, leading to a 20-fold increase in production. This might reflect sensitivity of the host strain to the antibiotic; over-expression of the immunity mechanism (in this case, the export machinery) apparently allowed for higher tolerance and therefore production of the antibiotic [48].

However the limitations of a *Streptomyces* host are more evident when attempting to express gene clusters isolated from phylogenetically more distant actinomycetes. Despite the success with the gene cluster for actagardine biosynthesis [56, 57] isolated from *Actinoplanes liguriae* (suborder Micromonosporineae), attempts to express the gene cluster for microbisporicin, isolated from *Microbispora corallina* (suborder Streptosporangineae) failed [58], although transcription of the biosynthetic genes was detected in *S. lividans* [59]. This might reflect the failure of the immunity mechanism to function effectively in the heterologous host and the existence of fail-safe systems that ensure production only occurs once immunity is in place [60]. A similar limitation was found initially when trying to express the gene cluster for planosporicin, isolated from *Planomonospora alba* (suborder Streptosporangineae). In this case, production of the antibiotic was achieved in *S. coelicolor* M1152 after removing the negative transcriptional regulation mediated by the gene encoding an anti-sigma factor present in the gene cluster; production was lower than in the natural producer, and was only observed on agar medium [61]. Heterologous production of both microbisporicin and planosporicin was readily obtained however when expressing the respective gene clusters in *Nonomuraea* sp. ATCC 39727, a closer relative of *Microbispora* and *Planomonospora* than *Streptomyces* species and from the same Streptosporangiaceae family [58, 62].

Perspectives

Here we have discussed the recent successes as well as failures of *S. coelicolor* as a heterologous host for the production of specialized metabolites derived from other actinomycetes. Overall, and without detracting from the use of other *Streptomyces* species (e.g., *S. avermitilis* [63]) and other actinomycetes (e.g., *Nonomuraea* [64]) as expression hosts, we believe that *S. coelicolor*, with its ease of culturing and genetic manipulation, has proven to be an extremely useful host for the heterologous production of actinomycete natural products. Whether the *S. coelicolor* strains described here can play a role in high volume commercial production remains to be seen (such applications might well require their further customized modification to achieve, for example, higher levels of precursor supply for different classes of compounds). Nevertheless, their value as discovery and analytical tools appears to be clear.

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6. Sources of therapeutically useful antibiotics. It is difficult to obtain accurate information about the number of marketed antibiotics obtained directly from, or derived from metabolites produced by, actinomycetes. Our figures were obtained by analysing the origin of the antibiotics listed on http://en.wikipedia.org/wiki/Timeline_of_antibiotics and classifying them into two categories, "fully synthetic" and "natural product related"; the last category was further divided according to the producing organism into three categories, "actinomycete", "other bacteria" and "fungi". The initial list contains 138 antibiotic formulations, of which 28 are based on a fully synthetic active pharmaceutical ingredient (API) (mostly quinolones and fluoroquinolones) and 110 contain APIs derived from natural products (80%). Of these 110, 48 formulations (44% of all natural product formulations; 35% of all formulations) contain metabolites isolated from actinomycetes (or semisynthetic derivatives of them), three are derived from other bacteria, and 59 contain APIs derived from metabolites produced by fungi (almost exclusively semisynthetic compounds derived from penicillins and cephalosporins). Therefore, 35% of all marketed antibiotic formulations contain an active ingredient derived, directly or indirectly, from an actinomycete. Our analysis suggests that all marketed APIs are derived from just 41 original molecules, 33 of which are natural products; only five of these 33 are produced by fungi and three by bacteria other than actinomycetes, leaving 25 original structures produced by actinomycetes. Thus actinomycetes appear to be the source of 61% of all original molecules and of 76% of the original natural product compounds developed for use in marketed antibiotic formulations
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