Uncovering production of specialized metabolites by *Streptomyces argillaceus*: Activation of cryptic biosynthesis gene clusters using nutritional and genetic approaches

Adriana Becerril¹,², Susana Álvarez¹, Alfredo F. Braña¹,², Sergio Rico³, Margarita Díaz³, Ramón I. Santamaría³, José A. Salas¹,², Carmen Méndez¹,²*

¹ Departamento de Biología Funcional e Instituto Universitario de Oncología del Principado de Asturias (I.U.O.P.A), Universidad de Oviedo, Oviedo, Spain, ² Instituto de Investigación Sanitaria de Asturias (ISPA), Oviedo, Spain, ³ Departamento de Microbiología y Genética, Instituto de Biología Funcional y Genómica, Consejo Superior de Investigaciones Científicas (CSIC)/Universidad de Salamanca, Salamanca, Spain

* cmendezf@uniovi.es

Abstract

Sequencing of *Streptomyces* genomes has revealed they harbor a high number of biosynthesis gene cluster (BGC), which uncovered their enormous potentiality to encode specialized metabolites. However, these metabolites are not usually produced under standard laboratory conditions. In this manuscript we report the activation of BGCs for antimycins, carotenoids, germicidins and desferrioxamine compounds in *Streptomyces argillaceus*, and the identification of the encoded compounds. This was achieved by following different strategies, including changing the growth conditions, heterologous expression of the cluster and inactivating the *adpAa* or overexpressing the *abrC3* global regulatory genes. In addition, three new carotenoid compounds have been identified.

Introduction

*Streptomyces* are Gram-positive bacteria with high GC content that show a differentiation cycle. They are known for being a prolific source of secondary metabolites (also referred here as specialized metabolites) [1], many of which show some kind of bioactivity [2]; for example, about 39% of bioactive compounds produced by microorganisms have a *Streptomyces* origin [3]. Traditionally, any *Streptomyces* strain was known to produce only one or a few specialized metabolites. However, since the first *Streptomyces* genome was sequenced it was brought to light that they harbored a higher number of biosynthesis gene clusters (BGC) than expected, which untapped their enormous potential to synthesize bioactive compounds [4]. During the last years, due to the development of next-generation sequencing technologies and bioinformatics tools such as AntiSMASH [5] and MIBiG [6] to identify candidate BGCs, a huge number of *Streptomyces* genome sequences has been made public, confirming their potential for...
synthesizing specialized metabolites [4,7]. Curiously, most of these metabolites are not usually identified under standard laboratory growth conditions even those compounds encoded by BGCs frequently found in Streptomyces genomes, which indicate that many of these BGCs are poorly or not expressed under those conditions. Therefore, several strategies have been developed to activate these silent BGCs and to identify the encoded compounds, which involves either genetic and/or culture conditions interventions [7–9].

Streptomyces argillaceus ATCC 12956 is known for producing the antitumor mithramycin, whose biosynthesis gene cluster has been cloned and thoroughly characterized [10]. However, no other specialized metabolite had been described in this strain until very recently, when its genome was sequenced and mined [11]. This has led to the identification of 31 putative BGCs for specialized metabolites none of them previously detected under standard laboratory growth conditions. Characterization of one of these clusters (cluster arp) led to the discovery of a new family of polyketide alkaloids named argimycins P [11,12]. Among the other identified BGCs, two were highly conserved and proposed to be involved in the biosynthesis of antimycins (cluster 27) and isorenieratene (cluster 26). However, their encoded compounds have not been identified so far in cultures of this strain, which suggests these BGCs are silenced or not normally expressed under standard laboratory culture conditions. Herein we report the use of nutritional and genetic approaches to activate expression of those BGCs in order to identify their encoded compounds. In addition, we report that inactivation of a global regulatory gene (an adpA-like) and overexpression of the S. coelicolor response regulator abrC3 led to the production of germicidins and to activate/increase production of several metabolites including the specialized metabolite desferrioxamine, respectively.

Material and methods

Strains, culture conditions, plasmids and DNA manipulations

*S. argillaceus* ATCC 12956, a mithramycin producer, was used as source of DNA. *S. argillaceus* and *S. albus* J1074 (ilv-1, sal-2) [13] were used as hosts for gene expression. R5A [14], SM17 [11], SM3, SM4, SM19, SM30 and SV2 media (S1 File) were used for production of secondary metabolites, following a two-step culture method [14]. *Escherichia coli* ET12567/pUB307 was used as donor in conjugation experiments [13]. When required, antibiotics were used at the following final concentrations: apramycin (25 μg/ml), thiostrepton (50 μg/ml), ampicillin (100 μg/ml), kanamycin (50 μg/ml), and nalidixic acid (25 μg/ml). A pKC505-based cosmid library of *S. argillaceus* chromosomal DNA [15] was used to identify cosmids containing the crta gene cluster, by using two DNA fragments obtained by PCR amplification using oligonucleotides genot25I-up and genot25I-rp (fragment I), and genot25D-up and genot25D-rp (fragment D) (S1 Table). DNA manipulations and transformations/conjugations were carried out using standard procedures [13,16]. Sequences analyses were performed using antiSMASH 4.1.0 [5] and BLAST [17]. Limits of each cluster were proposed after comparing genes (and their gene products) located at both ends with others in data-bases.

Plasmid constructs

pHLAbrC3c was constructed to express the *abrC3* gene from *S. coelicolor* [18] as follows: a PvuII DNA fragment containing the *oriT* and the apramycin resistance cassette was obtained from pIJ773 [19] and subcloned into the EcoRV of pHLAbrc3 [18].

pEM4T-sigmaC25 was generated to express *crtaQ* as follows: a 697 bp DNA fragment containing the *crtaQ* gene was amplified from cosmid pKC505-C25 as a BamHI-EcoRI fragment using oligonucleotides SigmaC25up and SigmaC25rp (S1 Table), and cloned into the same sites of pEM4T [20].
pΔadpAa was constructed to generate mutant *S. argillaceus ΔadpAa* as follows: a 1.8 kb DNA fragment containing the 3'-end of *adpAa* and downstream DNA region, was amplified using oligonucleotides *adpA*-5BglII and *adpA*-2R (S1 Table), digested with BglII and EcoRV, and subcloned into BamHI and EcoRV sites of pEFBAoriT [21], generating pEFBAadpAa2. Also, a 2.5 kb DNA fragment containing the 5'-end of *adpAa* was amplified using oligonucleotides *adpA*-3SpeI and *adpA*-3NsiI (S1 Table), digested with SpeI and NsiI and subcloned into the same sites of pEFBAadpAa2, upstream of the apramycin resistance cassette, generating pEFBAadpAa21. Finally, a hygromycin resistance cassette was isolated as a SpeI-NheI fragment from pLHyg [22] and subcloned into the XbaI site of pEFBAadpAa21.

**Extraction and analysis of secondary metabolites**

Culture samples (1 ml) were extracted with 1 volume of ethyl acetate and 1% formic acid (antimycins), *n*-butanol (desferrioxamine and germicidins) or methanol: tetrahydrofuran (THF) 1:1 and 1% of butylated hydroxytoluene (BHT) (carotenoid compounds). The organic extracts were evaporated *in vacuo* and dissolved in a small volume of DMSO:methanol (50:50). Samples were analyzed by reversed-phase chromatography on Acquity UPLC equipment with a BEH C18 column (1.7 mm, 2.1 x 100 mm; Waters, Milford, MA, USA), using acetonitrile and 0.1% trifluoroacetic acid (TFA) in water as eluent. Samples were eluted with 10% acetonitrile for 1 min, followed by a linear gradient from 10 to 90% acetonitrile over 7 min at a flow rate of 0.5 ml/min and a column temperature of 35˚C. For the carotenoid compounds, sample analyses were performed by reversed-phase chromatography on Alliance HPLC equipment with a SunFire C18 column (3.5 μm, 2.1 x 150 mm, Waters, Milford, MA, USA), using a mixture of methanol:acetonitrile (65:35) as eluent in isocratic conditions for 20 min, at a flow rate of 0.25 ml/min and a column temperature of 35˚C. Detection and spectral characterization of peaks were carried out with a photodiode array detector and Empower software (Waters). Mass analysis was performed by reversed-phase chromatography on Alliance HPLC equipment, coupled to a ZQ4000 mass spectrometer, with a SunFire C18 column (3.5 μm, 2.1 x 150 mm, Waters, Milford, MA, USA) with acetonitrile and 0.1% trifluoroacetic acid (TFA) in water as eluent. Samples were eluted with 10% acetonitrile for 4 min, followed by a linear gradient from 10 to 90% acetonitrile over 26 min at a flow rate of 0.25 ml/min and a column temperature of 35˚C. Detection and spectral characterization of peaks were carried out with a photodiode array detector and Empower software (Waters). Mass analyses were carried out as previously reported [23]. The identity of compounds was determined by dereplicating the extracts by LC-UV-LRMS and LC-HMRS against The Chapman & Hall Dictionary of Natural Products [24] and a database from Fundación Medina [25].

**Purification of germicidins**

*S. argillaceus ΔadpAa* was cultivated in five 2-liter Erlenmeyer flasks, each containing 400 ml of RSA medium, at 30˚C for five days. The cultures were centrifuged and the pellets were discarded. The supernatants were filtered, acidified by adding formic acid up to 1% and applied to a solid-phase extraction cartridge (Sep-Pak Vac C18, 10g, Waters). The retained material was eluted with a mixture of methanol and 0.05% TFA in water. A linear gradient from 0 to 100% methanol in 60 min, at 10 ml/min, was used. Analysis of fractions taken every 5 min indicated that the desired compounds eluted between 30 and 40 minutes. The corresponding fractions were pooled, evaporated *in vacuo* and redissolved in 2 ml of DMSO. The germicidins were purified by preparative HPLC using a SunFire C18 column (10 μm, 10 x 250 mm, Waters). The extract was chromatographed in isocratic conditions, first with a mixture of
acetonitrile and 0.1% TFA in water (55:45), and subsequently with a mixture of methanol and 0.1% TFA in water (75:25), at 5 ml/min. After every purification step, the collected material was diluted fourfold with water and then applied to a solid-phase extraction cartridge (Sep-Pak C18, Waters). The cartridge was washed with water, and the retained compounds were eluted with methanol and dried in vacuo. Once the purification was finished, the purified material was dissolved in a mixture of tert-butanol and water (1:1) and lyophilized, with a resulting yield of 5.2 mg.

Nucleotide sequences

Nucleotide sequences of gene clusters were deposited at the European Nucleotide Archive (EBI-ENA) under the accession numbers LT989885 (anta), LT989886 (crta), LT989884 (gcsA) and LT989883 (desa), and at Minimum Information about a Biosynthetic Gene Cluster (MIBIG) [6] under the accession numbers BGC0001455 (anta), BGC0001456 (crta), BGC0001454 (gcsA) and BGC0001453 (desa). The adpAa DNA region has been deposited at EBI-ANA under the accession number LS398138.

Results and discussion

Growth of *S. argillaceus* in different culture media resulted in activation of antimycins biosynthesis gene cluster 27 (anta)

Cluster 27 (anta; nucleotides 7920176–7946926 (Fig 1) shows high similarity to antimycins BGCs (S2 Table).

Antimycins are bioactive compounds characterized by a 9-membered dilactone core synthesized by a PKS-NRPS system bearing variations at C7 and C8, which is connected by an amide linkage to a 3-formamidosalicylic acid moiety [26] (Fig 2).

Antimycins BGCs are widespread among Actinobacteria: 73 antimycins BGC were identified in a recent study analyzing 1421 public genomes from selected genera of Actinobacteria [27]. All antimycins BGC identified so far show high synteny among them, and according to the number of genes and the presence/absence of the kynureninase antP and the phosphopantetheinyl transferase antQ they have been classified into four different classes: long-form (L-form), intermediate-form (I_P- or I_Q-form) and short-form (S-form) [26,27]. The anta cluster contains all genes required for the biosynthesis of antimycins (Fig 1; S2 Table): biosynthesis and activation of 3-formamidosalicylate starter unit (antaFGHIJKLNO); biosynthesis and modification of the 9-membered dilactone core (antaBCDE); and regulation (antaA). Since it lacks the antQ-like but contains an antP-like gene, the anta cluster can be classified as an I_P-form class.

Antimycins possess a characteristic absorption spectrum with maxima around 231 and 317 nm. However, no peaks with this spectrum were identified in *S. argillaceus* cultures in R5A medium (standard laboratory conditions). Since production of specialized metabolites is greatly influenced by changing culture conditions [28], production of antimycins was evaluated in a set of different media. This resulted in detection of antimycins production in five (SM3, SM4, SM19, SM30 and SV2) out of twenty five different media tested: four different pair of peaks were detected with the characteristic absorption spectrum of antimycins (peaks 1 to 8, Fig 3A), and with *m/z* values in positive mode of 507, 521, 535 and 549 (S1 Fig) that fit with the corresponding masses for antimycin A_4a/4b, A_3a/3b, A_2a/2b and A_1a/1b, respectively (Fig 2). Moreover, these antimycins showed the same retention times than antimycins A_1–A_4 previously identified in *S. albus* [29], confirming the identity of these compounds.
Activation of isorenieratene biosynthesis gene cluster 26 (crta) by its expression in a heterologous host

Cluster 26 (crta; nucleotides 7573410–7583894) shows high similarity to carotenoid isorenieratene BGCs (Fig 1; S3 Table). Carotenoids are terpenoids found in all photosynthetic organisms and also in some nonphototrophic organisms. They have different applications as food colorants, feed supplements, nutraceuticals and pharmaceuticals [30]. Many streptomycete strains contain isorenieratene BGCs that are usually silent. Only in a few cases activation of these BGCs has been achieved and production of their encoded compounds proved experimentally [31–33]. The crta cluster shows a similar gene organization to its homologous in \textit{S. avermitilis} [31]. It is organized in two divergent operons (crtaEIBV and crtaQYTU) that contain genes for the biosynthesis of carotenoid isorenieratene and biosynthesis intermediates [31] (Fig 1; S3 Table). In addition, it also contains \textit{crtaT} and \textit{crtaV} genes coding for a methyltransferase and a Rieske Non-Heme Iron-Dependent Oxygenase respectively, which have been also identified in other carotenoid BGCs. Noticeable in \textit{S. griseus} these genes have been shown to be dispensable for the biosynthesis of isorenieratene, the end product of the carotenogenic pathway in this strain [34]. The \textit{crta} BGC also contains a gene (\textit{crtaQ}) coding for an extracytoplasmic sigma factor-like that is not found in the \textit{crt} cluster of \textit{S. avermitilis}.

\textit{S. argillaceus} does not produce any carotenoid compound under standard laboratory conditions. It has been reported that actinomycetes can produce carotenoids in a light-induced manner or by the expression of an extracytoplasmic sigma factor [31]. Therefore, we tested if cultivation of \textit{S. argillaceus} under light conditions or overexpressing the extracytoplasmic
sigma factor-like crtQ under the control of the erythromycin resistant promoter (pEM4T-sigmaC25) could activate production of carotenoid compounds in this microorganism. However, production of carotenoid compounds was absent in all conditions (data not shown). In *S. griseus* IFO13350 activation of a cryptic carotenoid gene cluster was achieved by increasing its copy number [35]. Therefore we envisioned to increase the copy number of the crta cluster to activate it. To do so, we identified a cosmid containing the entire crta cluster by screening a *S. argillaceus* cosmid library with PCR probes generated from the 5'- and 3'-ends of the crta cluster (fragment-I and fragment-D). In this way, cosmid pKC505-C25 was selected containing the whole crta cluster. This cosmid was expressed in *S. argillaceus* and in *S. albus* that do not produce any pigmented compound under standard laboratory conditions. As control, the empty vector pKC505 was also introduced in both strains. No differences were observed between *S. argillaceus*-pKC505-C25 and the control strain (data not shown). On the contrary, cultures of *S. albus*-pKC505-C25 became yellow pigmented while those from the control strain remained unpigmented, being highest production of the yellow pigment detected using SM17 media (Fig 3B). HPLC chromatograms of organic extracts from these cultures revealed several peaks with UV-vis spectra corresponding to carotenoid compounds (peaks 9 to 15), which were absent in control cultures (Fig 3B). These carotenoids were identified by dereplication as 3,3’-dihydroxyprotoplene (peak 9), 3-hydroxyprotoplene (peak 12), leprotene (peak 14) and β-isorenieratene (peak 15) (Fig 2; S2 Fig). In addition, three new carotenoid compounds were
Fig 3. Production of specialized metabolites by *S. argillaceus*. (A) Antimycins (peaks 1 to 8): UPLC chromatogram at 230 nm of *S. argillaceus* grown in R5A (black) and in SM30 (red) media; (B) Carotenoids: (left) Erlenmeyer flasks containing cultures of *S. albus*-pKCS05 and *S. albus*-pKCS05-C25, and (right) HPLC chromatograms at 450 nm showing production of carotenoids (peaks 9 to 15) of *S. albus*-pKCS05 (black) and *S. albus*-pKCS05-C25 (red) in SM17; (C) Germicidins (peaks 16 and 17): UPLC chromatogram at 290 nm of *S. argillaceus* ΔadpA (red) and *S. argillaceus* wild type (black). M,
detected that based on their molecular formula are proposed to be the monomethylated derivatives of 3,3’-dihydroxyleproten e (peak 10) and 3-hydroxyleproten e (peak 13), and the dimethylated derivative of 3,3’-dihydroxyleproten e (peak 11), which is produced as the major compound (Figs 2 and 3B). The carotenogenic biosynthesis pathway of S. argillaceus would proceed through the synthesis of geranylgeranyl diphosphate units by CrtaE; formation of the C40 carbon chain phytoene by CrtaB; synthesis of lycopene through four desaturation steps by CrtaI; formation of β-carotene with two rings at both ends of the carbon chain by CrtaY; and synthesis of isorenieratene (leproten e) by a desaturation/methyltransferation mechanism in each ionone ring of β-carotene by CrtaU. Most of carotenogenic pathways in Streptomyces lead to isorenieratene as the final product [31,33], although in S. mediolani 3-hydroxy and 3,3’-dihydroxy derivatives of leproten e are the final products [36]. In the case of S. argillaceus this seems to be the dimethylated derivative of 3,3’-dihydroxyleproten e. Biosynthesis of this compound would require dihydroxylation of leproten e to generate 3,3’-dihydroxyleproten e, followed by its dimethylation. According to the genes identified in the crta cluster, candidate gene products to fulfill these functions could be the CrtaV Rieske Non-Heme Iron-Dependent Oxygenase and the CrtaT methyltransferase, respectively. 3,3’-dihydroxyleproten e was previously shown to have superior properties in preventing photo- and photooxidative damages in comparison to other carotenoids [37]. It remains to be determined if the three new compounds mono and dimethoxylated derivatives of leproten e here identified also show better properties.

Inactivation of the global regulatory gene adpAa resulted in activation of germicidin gene cluster

AdpA (A-factor-dependent protein A) is a key pleiotropic regulator first discovered in S. griseus where it acts as a positive transcriptional regulator of cell cycle differentiation and antibiotic production [38]. To determine the effect of inactivating adpA in S. argillaceus, it was necessary to clone that gene. Degenerated oligoprimer from conserved regions of adpA genes (S1 Table) were used to PCR amplified a homologous gene (adpAa) from S. argillaceus, which was later located in the S. argillaceus genome sequence [11] (nucleotides 2982405–2983677). This gene was mutated by replacing it by an apramycin resistance cassette that was inserted in the same direction of transcription than the target gene, generating mutant S. argillaceus ΔadpAa. The replacement event was confirmed by PCR (Fig 4): using oligonucleotides SCR-F and adpAa CR (S1 Table), a 2.1 kb DNA fragment was amplified from the wild type strain while a 2.4 kb was amplified from the mutant, confirming the replacement event.

Analysis of organic extracts of cultures of this mutant strain (S. argillaceus ΔadpAa) revealed that production of mithramycin strongly decreased (peak M, Fig 3C), but noticeable two compounds with similar retention times were accumulated (peaks 16 and 17, Fig 3C). These compounds were not detected in the wild type strain (Fig 3C). Purification of each single compound was attempted but not achieved, being only possible to purify them as a mixture. Both compounds displayed a maximum at ca. 290 nm in the UV (DAD) spectrum and m/z values of 183 [M+H]+ what suggested being isomers. The HRMS information (S3 Fig) rendered a molecular formula of C10H14O3 based on the observed ion [M+H]+ at 183.1019 (calcd. for C10H15O3+ = 183.1016). Such formula and the observed UV (DAD) spectrum were compatible with germicidin B, isogermicidin B and germicidin C. The 1H NMR and the HSQC spectra
(S4 Fig; S6 Table) revealed by comparison with reported data [39] that the sample comprises a mixture of germicidin B and germicidin C (Fig 2) in a ratio ca. 2.4:1. These compounds are known to have an inhibitory effect on germination of Streptomyces spores [40], and it has been proposed to play a role in coordinating the germination process within a spore population [41]. In S. coelicolor it has been shown that biosynthesis of germicidins only requires a Type III PKS (Gc) encoded by sco7221, in addition to enzymes from the fatty acid pathway to supply acyl-ACPs [39,42]. The AntiSMASH analysis of S. argillaceus genome did not identify those genes, but a deeper analysis of its genome allowed to identifying a gene (nucleotides 982748 to 983933) coding for a Type III PKS, which showed high similarity to germicidin synthases and was named gcsA (S4 Table). In addition, homologous genes to those involved in supplying acyl-ACPs (fabD, fabH and acpA) (Fig 1; S4 Table) were also identified in S. argillaceus genome, about 1.5 Mb upstream of gcsA. AdpA is considered a transcriptional activator of antibiotic production [38]. However, results shown here indicate that it can also play a negative role in production of specialized metabolites. Very recently it has been reported that a cryptic oviedomycin BGC in S. ans ochromogenes was also activated by disrupting an adpA gene [43]. The activation of the germicidins production represents the second example of using adpA inactivation as an approach to activate a silent pathway, and confirms this new strategy as a possible choice to be applied in genomics-driven discovery of natural products.

**Detection of desferrioxamine production by overexpressing the response regulator abrC3**

AbrC3 is a response regulator that has been shown to positively control actinorhodin and undecylprodiginine production, and morphological differentiation in S.coelicolor [18]. Consequently, we tested the possibility of activating and/or enhancing production of specialized metabolites in S. argillaceus by overexpressing abrC3. After introducing abrC3 (pHJLAbrC3c)
into *S. argillaceus*, the recombinant strain was cultivated in R5A and SM17 media and cultures were extracted with ethyl acetate or butanol. Extracts were analyzed by HPLC-MS and compared with those obtained from cultures of a control strain containing the empty vector (pHJL401c). Cultures of *S. argillaceus*-pHJLAbc3c in SM17 medium and extracted with butanol showed more than nine compounds that were either de novo produced or produced in higher amounts than in the control strain (Fig 3D). Dereplication of these samples allowed to identifying a compound that increased about 55% in relation to the control culture, eluted at 5.16 min and with an m/z value 561.3603 (Fig 3D; S5 Fig), for which the suggested molecular formula was C22H48N6O8 that corresponds to desferrioxamine B (Fig 2), a linear trihydroxamic acid siderophore with clinical application [44]. AntiSMASH analysis of *S. argillaceus* genome revealed that cluster 13 (desa; nucleotides 2970011–2977244) showed high similarity and synteny to the desferrioxamine gene cluster from *S. coelicolor* [45] (Fig 1; S5 Table). The desa cluster contains all genes required for the biosynthesis of desferrioxamine B [44] (S5 Table): a pyridoxal 5’-phosphate (PLP)-dependent lysine decarboxylase (desaA), a FAD-dependent monooxygenase (desaB), an acyl coenzyme A transferase (desaC) and a NTP-dependent siderophore synthetase (desaD). In addition, upstream of these four genes two genes are localized (desaE and desaF) that have been proposed to be involved in transporting cyclic trishydroxamate and utilization of iron from cyclic tris-hydroxamate iron-siderophore complexes, in *S. coelicolor* [45].

In conclusion, we have been able to activate four BGCs from *S. argillaceus*, proving their functionality. In addition, we have identified the putative encoded compounds. Further experiments (cluster inactivation and/or heterologous expression) would definitively proof the link between clusters anta, gcs and desa to the identified compounds. The selected BGCs are widely distributed among streptomycetes, but only in a few cases their functionality has been proved and the encoded compounds have been identified in cultures of those strains. The activation of these BGCs was achieved using different approaches: changing the cultivation conditions (for the antimycins anta cluster), expressing the cluster in a heterologous host (for the carotenoids crta cluster), and inactivating adpAa or overexpressing abrC3 global regulatory genes (for the germicidins gcsA and desferrioxamine desa clusters, respectively). In addition, three new carotenoid compounds have been identified. Results shown here will contribute to extend the knowledge about the specialized metabolism of the *S. argillaceus* strain, which could be used to activate and identify other specialized metabolites and to improve and generate new compounds by metabolic engineering.

**Supporting information**

S1 Fig. HPLC-MS analyses of compounds in peaks identified in Fig 3A.  
(DOCX)

S2 Fig. MS analyses of compounds in peaks identified in Fig 3B.  
(DOCX)

S3 Fig. HRMS spectra of germicidins.  
(DOCX)

S4 Fig. 1H NMR and HSQC spectra of germicidins (CDCl3, 500 MHz).  
(DOCX)

S5 Fig. MS analysis of compound in peak 18 in Fig 3D.  
(DOCX)
S1 File. Composition of production media. (DOCX)

S1 Table. Oligonucleotides used in this work. (DOCX)

S2 Table. Functions of gene products for antimycin gene cluster (anta). (DOCX)

S3 Table. Functions of gene products for isorenieratene gene cluster (crta). (DOCX)

S4 Table. Functions of gene products for germicidin gene cluster (gcsA). (DOCX)

S5 Table. Functions of gene products for desferrioxamine gene cluster (desa). (DOCX)

S6 Table. NMR data of germicidins B and C. (DOCX)

Acknowledgments

We thank Dr. Carlos Olano for helping in managing DNA sequences, and Dr. Fernando Reyes from Fundación Medina for technical support in structural analyses of compounds.

Author Contributions

Conceptualization: Margarita Díaz, Ramón I. Santamaría, José A. Salas, Carmen Méndez.

Funding acquisition: Carmen Méndez.

Investigation: Adriana Becerril, Susana Álvarez, Alfredo F. Braña.

Methodology: Sergio Rico.

Supervision: Carmen Méndez.

Writing – original draft: Carmen Méndez.

Writing – review & editing: Carmen Méndez.

References

1. van Keulen G, Dyson PJ. Production of specialized metabolites by Streptomyces coelicolor A3(2). Adv Appl Microbiol 2014; 89:217–266. https://doi.org/10.1016/B978-0-12-800259-9.00006-8 PMID: 25131404

2. Barka EA, Vatsa P, Sanchez L, Gaveau-Vaillant N, Jacquard C, Klenk HP, et al. Taxonomy, physiology, and natural products of Actinobacteria. Microbiol Mol Biol Rev 2016; 80:1–4. https://doi.org/10.1128/MMBR.00019-15 PMID: 26609051

3. Bérody J. Thoughts and facts about antibiotics: Where we are now and where we are heading. J Antibiot (Tokyo) 2012; 65: 385–395.

4. Nett M, Ikeda H, Moore BS. Genomic basis for natural product biosynthetic diversity in the actinomycetes. Nat Prod Rep 2009; 26:1362–1384. https://doi.org/10.1039/b817069h PMID: 19844637

5. Weber T, Blin K, Duddela S, Krug D, Kim HU, Brucoleri R, et al. AntiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Res 2015; 43:W237–W243. https://doi.org/10.1093/nar/gkw437 PMID: 25948579

6. Medema MH, Kottermann R, Yilmaz P, Cummings M, Biggins JB, Blin K, et al. Minimum information about a biosynthetic gene cluster. Nat Chem Biol 2015; 11:625–631. https://doi.org/10.1038/nchembio.1890 PMID: 26284661
7. Rutledge PJ, Challis GL. Discovery of microbial natural products by activation of silent biosynthetic gene clusters. Nat Rev Microbiol 2015; 13:509–523. https://doi.org/10.1038/nrmicro3496 PMID: 26119570
8. Olano C, Mendez C, Salas JA. Strategies for the Design and Discovery of Novel Antibiotics using Genetic Engineering and Genome Mining. In: Villa TG, Veiga-Crespo P editors. Antimicrobial compounds: current strategies and new alternatives. Springer: Berlin. Heidelberg, Germany; 2014. pp. 1–25.
9. Zhang MM, Wong FT, Wang Y, Luo S, Lim YH, Heng E, et al. CRISPR-Cas9 strategy for activation of silent Streptomyces biosynthetic gene clusters. Nat Chem Biol 2017; 13:607–609.
10. Méndez C, González-Sabin J, Morís F, Salas JA. Expanding the chemical diversity of the antitumoral compound mithramycin by combinatorial biosynthesis and biocatalysis: the quest for mithrolags with improved therapeutic window. Planta Med 2015; 81:1326–1338. https://doi.org/10.1055/s-0035-1557876 PMID: 26393942
11. Ye S, Molloy B, Braña AF, Zabala D, Olano C, Cortés J, et al. Identification by genome mining of a type I polyketide gene cluster from Streptomyces argillaceus involved in the biosynthesis of pyridine and piperidine alkaloids argimycins P. Front Microbiol 2017; 8:194. https://doi.org/10.3389/fmicb.2017.00194 PMID: 28239372
12. Ye S, Braña AF, González-Sabin J, Morís F, Olano C, Salas JA et al. New insights into the biosynthesis pathway of polyketide alkaloid argimycins P in Streptomyces argillaceus. Front Microbiol 2018; 9:252. https://doi.org/10.3389/fmicb.2018.00252 PMID: 29503641
13. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA. Practical Streptomyces Genetics. Norwich: The John Innes Foundation, UK; 2000.
14. Fernández E, Weibbach U, Sánchez Reillo C, Braña AF, Méndez C, Rohr J, et al. Identification of two genes from Streptomyces argillaceus encoding two glycosyltransferases involved in the transfer of a disaccharide during the biosynthesis of the antitumor drug mithramycin. J Bacteriol 1998; 180:4929–4937. PMID: 9733697
15. Lombó F, Blanco G, Fernández E, Méndez C, Salas JA. Characterization of Streptomyces argillaceus genes encoding a polyketide synthase involved in the biosynthesis of the antitumor mithramycin. Gene 1996; 172:87–91. PMID: 8654997
16. Sambrook J, Russell DW. Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor; 2001.
17. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997; 25:3389–3402. PMID: 9254694
18. Rico S, Santamaría RI, Yepes A, Rodríguez H, Laing E, Bucca G, et al. Deciphering the region of Streptomyces coelicolor AbrC3, a positive response regulator of antibiotic production. Appl Environ Microbiol 2014; 80:2417–2428. https://doi.org/10.1128/AEM.00108-11 PMID: 24509929
19. Gust B, Challis GL, Fowler K, Kieser T, Chater KF. PCR-targeted Streptomyces gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci U S A 2003; 100:1541–1546. https://doi.org/10.1073/pnas.0337542100 PMID: 12656303
20. Menéndez N, Nur-e-Alam M, Fischer C, Braña AF, Salas JA, Rohr J, et al. Deoxysugar transfer during chromomycin A3 biosynthesis in Streptomyces griseus subsp. griseus: new derivatives with antitumor activity. Appl Environ Microbiol 2006, 72:167–177. https://doi.org/10.1128/AEM.72.1.167-177.2006 PMID: 16391039
21. Horna DH, Gómez C, Olano C, Palomino-Schätzlein M, Pineda-Lucena A, Carabajo RJ, et al. Biosynthesis of the RNA polymerase inhibitor streptolydygin in Streptomyces lydicus: tailoring modification of 3-methyl-aspartate. J Bacteriol 2011; 193:2647–2651. https://doi.org/10.1128/JB.00108-11 PMID: 21398531
22. Olano C, Wilkinson B, Sánchez C, Moss SJ, Sheridan R, Math V et al. Biosynthesis of the angiogenesis inhibitor borrelidin by Streptomyces parvulus Tü4055: cluster analysis and assignment of functions. Chem Biol 2004; 11:97–97. https://doi.org/10.1016/j.chembiol.2003.12.018 PMID: 15112996
23. Lombó F, Gibson M, Greenwell L, Braña AF, Rohr J, Salas JA, et al. Engineering biosynthetic pathway for deoxysugars: branched chain sugar pathways and novel derivatives from the antitumor tetracenomycin. Chem. Biol. 2004; 11:1709–1718. https://doi.org/10.1016/j.chembiol.2004.10.007 PMID: 15610855
24. Buckingham J. Dictionary of Natural Products on DVD, version 22.2. London: Taylor & Francis/CRC Press; 2013.
25. Pérez-Victoria I, Martín J, Reyes F. Combined LC/UV/MS and NMR strategies for the dereplication of marine natural products. Planta Med 2016; 82:857–871. https://doi.org/10.1055/s-0042-101763 PMID: 27002401
Activation of gene clusters in *Streptomyces argillaceus*

26. Liu J, Zhu X, Kim SJ, Zhang W. Antimycin-type depsipeptides: discovery, biosynthesis, chemical synthesis, and bioactivities. Nat Prod Rep 2016; 33:1146–1165. doi:10.1039/c6np00004e PMID: 27307039

27. Joynt R, Seipke RF. A phylogenetic and evolutionary analysis of antimycin biosynthesis. Microbiology 2018; 164:28–39. doi:10.1099/mic.0.000572 PMID: 29119164

28. Bode HB, Bethe B, Höfs R, Zeeck A. Big effects from small changes: possible ways to explore nature’s chemical diversity. ChemBioChem 2002; 3:619–627. doi:10.1002/1439-7633(20020703)3:7<619:AID-CBIC619>3.0.CO;2-9 PMID: 12324995

29. Olano C, García I, González A, Rodríguez M, Rozas D, Rubio J, et al. Activation and identification of five clusters for secondary metabolites in *Streptomyces j1074*. Microb Biotechnol 2014; 7:242–256 See comment in PubMed Commons below. https://doi.org/10.1111/1751-7915.12116 PMID: 24593309

30. Schieber A, Weber F. Carotenoids. In: Carle R and Schweigert R editors. Handbook on Natural Pigments in Food and Beverages: Industrial applications for improving food color. Elsevier limited; 2016, pp 101–123.

31. Takano H, Asker D, Beppu T, Ueda K. Genetic control for light-induced carotenoid production in non-phototrophic bacteria. J Ind Microbiol Biotechnol 2006; 33:88–93. doi:10.1007/s10295-005-0005-z PMID: 16091943

32. Iftime D, Kulik A, Hárta T, Rohrer S, Niedermeyer TH, Stegmann E, et al. Identification and activation of novel biosynthetic gene clusters by genome mining in the kirromycin producer *Streptomyces collinus* Tü 365. J Ind Microbiol Biotechnol 2016; 43:277–291. doi:10.1007/s10295-015-1685-7 PMID: 26433383

33. Myronovskyi M, Tokovenko B, Brótz E, Rücker C, Kalinowski J, Luzhetskyy A. Genome rearrangements of *Streptomyces j1074* lead to the carotenoid gene cluster activation. Appl Microbiol Biotechnol 2014; 98:795–806. doi:10.1007/s00253-013-5440-6 PMID: 2433797

34. Krügel H, Krubasik P, Weber K, Saluz HP, Sandmann G. Functional analysis of genes from *Streptomyces griseus* involved in the synthesis of isorenieratene, a carotenoid with aromatic end groups, revealed a novel type of carotenoid desaturase. Biochim Biophys Acta 1999; 1439:57–64. PMID: 10395965

35. Lee HS, Ohnishi Y, Horinouchi S. A sigmaB-like factor responsible for carotenoid biosynthesis in *Streptomyces griseus*. J Mol Microbiol Biotechnol 2001; 3:95–101. PMID: 11200234

36. Arcamone F, Camerino B, Cotta E, Franceschi G, Grein A, Penco S, et al. New carotenoids from *Streptomyces mediolani*s. sp. Experientia 1969; 25:241–241. PMID: 5781524

37. Wagener S, Völker T, De Spirt S, Ernst H, Stahl W. 3,3’-Dihydroxyisorenieratene and isorenieratene prevent UV-induced DNA damage in human skin fibroblasts. Free Radic Biol Med 2012; 53:457–463. https://doi.org/10.1016/j.freeradbiomed.2012.05.022 PMID: 22634149

38. Ohnishi Y, Yamazaki H, Kato JY, Tomono A, Horinouchi S. AdpA, a central transcriptional regulator in the A-factor regulatory cascade that leads to morphological development and secondary metabolism in *Streptomyces griseus*. Biosci Biotechnol Biochem 2005; 69:431–439. See comment in PubMed Commons below https://doi.org/10.1271/bbb.69.431 PMID: 15784968

39. Song L, Barona-Gomez F, Corre C, Xiang L, Udward DW, Austin MB, et al. Type III polyketide synthase β-ketoacyl-ACP starter unit and ethylmalonyl-CoA extender unit selectivity discovered by *Streptomyces coelicolor* genome mining. J Am Chem Soc 2006; 128:14754–14755. doi:10.1021/ja065247w PMID: 17105255

40. Petersen F, Zähner H, Metzger JW, Freund S, Hummel RP. Germicidin, an autoregulatory germination inhibitor of *Streptomyces viridochromogenes* NRRL B-1551. J Antibiot (Tokyo) 1993; 46:1126–1138.

41. Čihák M, Kameník Z, Šmídová K, Bergman N, Benada O, Kofroňová O, et al. Secondary Metabolites Produced during the Germination of *Streptomyces coelicolor*. Front Microbiol 2017; 8:2495. doi:10.3389/fmicb.2017.02495 PMID: 29326665

42. Chemler JA, Buchholz TJ, Geders TW, Akey DL, Rath CM, Chipala GE, et al. Biochemical and structural characterization of germicidin synthase: analysis of a type III polyketide synthase that employs acyl-ACP as a starter unit donor. J Am Chem Soc 2012; 134:7359–7366. https://doi.org/10.1021/ja2112228 PMID: 22480290

43. Xu J, Zhang J, Zhuo J, Li Y, Tian Y, Tan H. Activation and mechanism of a cryptic oviedomycin gene cluster via the disruption of a global regulatory gene, adpA, in *Streptomyces ansaochromogenes*. J Biol Chem 2017; 292:19708–19720. https://doi.org/10.1074/jbc.M117.809145 PMID: 28972164

44. Challis GL. A widely distributed bacterial pathway for siderophore biosynthesis independent of nonribosomal peptide synthetases. ChemBioChem 2005; 6:601–611. https://doi.org/10.1002/cbic.200400283 PMID: 15719346
45. Barona-Gómez F, Wong U, Giannakopoulos AE, Derrick PJ, Challis GL. Multiple biosynthetic and uptake systems mediate siderophore-dependent iron acquisition in *Streptomyces coelicolor* A3(2) and *Streptomyces ambofaciens* ATCC 23877. Microbiology 2006; 152:3355–3366. https://doi.org/10.1099/mic.0.29161-0 PMID: 17074905