The gamma-butyrolactone system from *Streptomyces filipinensis* reveals novel clues
to understand secondary metabolism control.

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Running title: The GBL system from *S. filipinensis*
ABSTRACT: Streptomyces γ -butyrolactones (GBLs) are quorum sensing communication signals triggering antibiotic production. The GBL system of *S. filipinensis*, the producer of the antifungal agent filipin, has been investigated. Inactivation of sfbR, a GBL receptor, resulted in a strong decreased production of filipin while deletion of sfbR2, a pseudo-receptor, boosted it, in agreement with lower or higher transcription of filipin biosynthetic genes respectively. Noteworthy, none of the mutations affected growth or morphological development. While no ARE (autoregulatory element)-like sequences were found in the promoters of filipin genes, suggesting an indirect control of production, five ARE sequences were found in five genes of the GBL cluster, whose transcription has been shown to be controlled by both SfbR and SfbR2. *In vitro* binding of recombinant SfbR and SfbR2 to such sequences indicated that such control is direct. Transcription start points were identified by 5′-rapid amplification of cDNA ends, and precise binding regions were investigated by DNase I protection studies. Binding of both regulators took place in the promoter of target genes and at the same sites. Information content analysis of protected sequences in target promoters yielded an 18-nucleotide consensus ARE sequence. Quantitative transcriptional analyses revealed that both regulators are self-regulated and repress each other transcription as well as that of the remaining target genes. Unlike other GBL receptor homologues, SfbR activates its own transcription, while SfbR2 has a canonical auto-repression profile. Additionally, SfbR2 was found here to bind antifungal antimycin A as a way to modulate its DNA-binding activity.

IMPORTANCE: *Streptomyces* GBLs are important signalling molecules triggering antibiotic production in a quorum sensing dependent manner. We have characterized the
GBL system from *S. filipinensis*, finding that two key players of this system, the GBL receptor and the pseudo-receptor, counteract each other transcription for the modulation of filipin production, and that such control over antifungal production involves an indirect effect on the transcription of filipin biosynthetic genes. Additionally, both regulators bind the same sites, are self-regulated and repress the transcription of three other genes of the GBL cluster, including that of the GBL synthase. Contrary to all the GBL receptors known, SfbR activates its own synthesis. Moreover, the pseudo-receptor was identified as the receptor of antimycin A, thus extending the number of examples supporting the signalling effects of antibiotics in *Streptomyces*. The intricate regulatory network depicted here should provide important clues to understand the regulatory mechanism governing secondary metabolism.

**KEYWORDS:** ARE sequence, GBLs, filipin, polyene macrolide, *Streptomyces*
INTRODUCTION

Streptomyces are soil-dwelling bacteria that undergo a rather complex differentiation process which is usually accompanied by antibiotic and other secondary metabolite-bioactive molecules production such as anticancer agents, immunosuppressants or antihelmintic agents, among others (1). The onset of this process is often controlled by small extracellular signalling molecules (autoregulators) that coordinate the population behaviour at nanomolar concentrations, hence they have been sometimes regarded as bacterial hormones. Several classes of autoregulators have been identified in Streptomyces including furanes (2), butenolides (3), butanediols (4), and diketopiperazines (5), but the most thoroughly studied group is that of γ-butyrolactones (GBLs). These share a 2,3-disubstituted GBL scaffold with a variable C2 side-chain which is species specific (6) except for one example, SVB1 from S. venezuelae which is identical to SCB3 from S. coelicolor (7).

GBLs elicit secondary metabolite biosynthesis by regulating the DNA binding activity of cognate receptor proteins. In a normal scenario, the GBL interacts with its specific receptor protein and releases its repression of target genes, thus activating gene expression (6). Target genes are generally involved in secondary metabolism, but occasionally also in morphological differentiation (8). Most GBL receptors target cluster situated regulatory genes linked to secondary metabolite gene clusters or the global regulatory gene adpA (9), hence given the wide influence of such regulators (10,11) effects on gene expression are in most occasions result of regulatory cascade mechanisms (12). Although most GBL receptors act as transcriptional repressors (13), some have been...
described to act as positive regulators, such as SpbR from *S. pristinaespiralis* (14) or SprA from *S. chattanoogensis* (15), and others have both repressor and activator activities depending on the availability of GBL, such as JadR3 in *S. venezuelae* (7).

Our knowledge about GBL biosynthesis is limited, but it seems clear that a protein homologous to AfsA, the key enzyme in A-factor biosynthesis in *S. griseus* is required for the formation of GBLs (16,17). AfsA catalyzes the first step of the biosynthesis, the condensation of dihydroxyacetone phosphate (a glycerol derivative) and a β-ketoacid forming a fatty acid ester, which is converted into A-factor by three steps of dephosphorylation, aldol condensation and reduction (18,19).

In *Streptomyces* genomes *afsA*-like genes are commonly found next or near to GBL receptor encoding genes, and located in the vicinity of or within antibiotic biosynthetic gene clusters. In general, the receptor acts as a repressor of the biosynthesis of its specific GBL synthase and regulates its own synthesis, forming a negative feedback loop, in addition to modulating the secondary metabolism (9). Many *Streptomyces* spp. additionally possess a range of auxiliary regulators, encoded within the same GBL gene cluster, that modulate the activity of this central circuit (20). Among such auxiliary regulators are pseudo-receptors, homologues of GBL receptor proteins without the ability to bind the GBL ligand, but with the capacity to bind other ligands such as antibiotics (21-23), and a role in the regulation of GBL production (23-25).

*S. filipinensis* produces a family of polyene polyketide macrolides known as filipins, which have broad spectrum antifungal activity, being filipin III the major component. Although filipin III has been reported to be produced by other strains, *S. filipinensis* is the industrial producer of the antifungal, being its production
substantially higher than in other strains (26). Despite being a polyene and having a potent antifungal activity derived from its interaction with the ergosterol of fungal membranes, this pentaene also shows a rather high affinity for cholesterol, which makes it useless in human therapy. Nonetheless, it is widely used for the detection and the quantitation of cholesterol in biological membranes (27) and as a tool for the diagnosis of Niemann-Pick type C disease (28). Its biosynthetic pathway in *S. filipinensis* has recently been discovered (26), and besides a recent study on the mechanism of phosphate control of filipin biosynthesis (29), there is an absolute lack of knowledge on regulatory mechanisms of antibiotic production in this bacterium. It was therefore, of great interest to study the GBL system of *S. filipinensis* and its role on filipin production.

**RESULTS AND DISCUSSION**

**Cloning of a γ-butyrolactone gene cluster in *S. filipinensis***.

The GBL gene cluster was identified by hybridization using a cosmid library (26) and a 149 bp probe obtained by PCR amplification of *S. filipinensis* chromosomal DNA with degenerate oligonucleotides derived from conserved stretches of the N-terminal region of several GBL receptors (see “materials and methods”). Once a gene homologous to GBL receptors was identified (*sfbR*), the remaining genes of the cluster were identified by chromosome walking. The deduced gene organization within this region is shown in Fig 1A.

**In silico analysis and arrangement of genes.**
Computer-assisted analysis of the 11542 bp sequenced region revealed nine complete open reading frames (ORFs). Among these genes, two GBL receptor-like encoding genes were identified and named sfbR and sfbR2 (for \textit{S. filipinensis} \(\gamma\)-butyrolactone receptor) and a gene homologous to GBL synthase encoding genes (sfbA). Table S1 shows the deduced functions of all these genes.

At the left end of the cluster, sfb1 encodes a large size SARP-like regulator sharing the same domain architecture as AfsR from \textit{S. coelicolor} (30) and showing a 39% identity along its full length. Downstream and in the same orientation lies sfb2 that encodes a putative regulator of the StrR family showing 49% identity with KasT, a regulator encoded by the kasugamycin biosynthetic cluster of \textit{S. kasugaensis} (31). Both sfb2 and kasT contain the rare leucine TTA codon, which has been correlated with regulatory genes involved in antibiotic production (32). sfbR is situated downstream, and encodes a protein with convincing similarity to GBL receptors from other \textit{Streptomyces} spp. The highest scores were with SpbR from \textit{S. pristinaespiralis} (14) (57% identity) and AvaR1 from \textit{S. avermitilis} (3) (56% identity). Fig. S1 shows an alignment with other GBL receptors.

Downstream from sfbR is sfb4, a cytochrome P450 monooxygenase-encoding gene. It shows highest similarity to SnbU from \textit{S. pristinaespiralis} (33) (58% identity), Orf16* from \textit{S. fradiae} (34) (57% identity) and Cyp17 from \textit{S. avermitilis} (35) (56% identity). Interestingly, in all these four cases the genes for cytochrome monooxygenases are adjacent to genes coding for GBL receptors, suggesting that these enzymes may be involved in the biosynthesis of butyrolactone, although their role has only been demonstrated in Cyp17, which is implicated in the biosynthesis of avenolide, a signaling
molecule of the butenolide type (3). sfb4 also contains the rare leucine TTA codon, a feature that shares with other cytochrome P450 monoxygenase-encoding genes from GBL gene clusters such as orf16* from S. fradiae (34) or tsuB from S. tsukubaensis (36).

sfb5 is located downstream, it encodes a putative short-chain dehydrogenase/reductase with similarity to Orf4 protein from Streptomyces sp. SBI034 (37) (51% identity) and ScbC from S. coelicolor (38) (50% identity). These enzymes have been proposed to be nucleoside-diphosphate-sugar epimerases involved in GBL biosynthesis, thus it is conceivable that Sfb5 could have a role in the biosynthesis of S. filipinensis GBL. The two genes that follow are oriented convergently (Fig. 1A), sfb6 that encodes a hypothetical protein of unknown function, and sfb7 whose coding strand is in the opposite strand of all the identified genes and encodes a small size SARP-like regulator. This protein is highly similar to other SARPs belonging to GBL clusters such as BulY from S. tsukubaensis (36), FarR4 from S. lavendulae (39) and SgvR2 from S. griseoviridis (40) (66, 65 and 65 % identity respectively).

Downstream from sfb7 is sfbR2 (Fig. 1A), which encodes a putative GBL pseudo-receptor (see below). It showed highest similarity scores with PapR5 from S. pristinaespiralis (33) (51 % identity) and TylQ from S. fradiae (34) (47 % identity). Fig. S2 shows an alignment of SfbR2 with other GBL pseudo-receptors. Downstream and in the same orientation lies sfbA, whose product shows high similarity with GBL synthases such as SrrX from S. rochei (41) (57% identity) or Lct9 from S. rishiriensis (42) (55% identity). Fig. S3 shows an alignment of SfbA with other GBL synthases.

When compared with other GBL gene clusters, no obvious gene synteny is observed. Although the GBL receptor and pseudo-receptor tend to be clustered with the
GBL biosynthesis gene/s (6) (Fig. 1B), many exceptions have been described. Thus, *S. avermitilis* or *S. fradiae* GBL gene clusters lack GBL synthase-encoding genes and harbour an acyl-CoA oxidase coding gene (*aco*) instead that possibly substitutes GBL synthase (3,34). Another exception is the *S. coelicolor* GBL gene cluster where the *scbR2* pseudo-receptor gene is separated from the GBL synthase and the receptor genes by the coelimycin *cpk* cluster (Fig. 1B) (43). Hence, it seems there are no unifying principles among GBL gene clusters.

**SfbR is a putative GBL receptor whereas SfbR2 is a pseudo-receptor.**

Both GBL receptors and pseudo-receptors belong to the TetR family of regulators. These comprise a conserved helix-turn-helix DNA-binding motif at the N-terminal region and a variable C-terminal domain involved in ligand binding (44,45). The latter contains a highly conserved tryptophan residue which is involved in ligand binding (46). Interestingly, SfbR and SfbR2 share a 34% identity and both contain such conserved tryptophan residue (W123 in SfbR and W128 in SfbR2 (Figs. S1 and S2)).

Despite their structural similarity, when compared with counterparts in the databases, SfbR clusters with genuine GBL receptors whereas SfbR2 does it with pseudo-receptors. Fig. 2 shows a phylogenetic analysis with whole amino acid sequences. Besides, SfbR has a calculated pI of 6.54, a slightly acidic-neutral value that is characteristic of genuine receptors, whereas that of SfbR2 is 8.74, a basic value common in pseudo-receptors (39). Most of the proteins analysed follow such paradigm but there are a few exceptions (indicated in red in Fig. 2). Noteworthy, MmfR, an authentic methylenomycin furan receptor from *S. coelicolor* (47) with a pI value of 5.99, as expected, but which clusters with pseudo-receptors, and ScbR2 an *S. coelicolor* GBL
pseudo-receptor (21) which clusters with pseudo-receptors, as expected, but which has a slightly acidic pI value of 5.85.

Inactivation of sfbR reduces filipin production while sfbR2 deletion increases it.

To assess the function of sfbR or sfbR2, we deleted them by using the REDIRECT gene replacement technology as indicated in “materials and methods”. Double-crossover mutants that had lost sfbR were screened by apramycin resistance whereas those lacking sfbR2 were selected by their resistance to spectinomycin. All mutants were further verified by PCR analysis (Fig. S4).

In order to study the effect that the inactivation of the sfbR and sfbR2 genes had on the production of filipin, fermentation broths produced by the new mutant strains, when grown in YEME medium, were extracted with methanol and analysed for the presence of filipin III (the major component of the filipin complex). Results indicated that *S. filipinensis ΔsfbR* was impaired in filipin production, reaching ca. 50% of the production observed in the parental strain (Fig. 3A), thus suggesting that SfbR is an activator of filipin biosynthesis. On the contrary, *S. filipinensis ΔsfbR2* behaved as a filipin overproducer, reaching about two-fold of the filipin produced by the wild type strain at 72h of growth (Fig. 3A), which suggested that SfbR2 is a negative regulator of antifungal biosynthesis. Interestingly, none of the mutations affected growth or morphological development on solid medium (not shown), and the growth curves of the mutants closely resembled the growth of their parental strain (Fig. 3A). This indicates that neither SfbR nor SfbR2 affect significantly primary metabolism under the conditions used.
Gene complementation restores filipin biosynthesis in the mutants.

To confirm that the gene deletions were directly responsible for the effects observed on filipin III production, we complemented both mutants with the corresponding gene. For that purpose, we introduced one copy of the gene including its promoter region into the genome of the mutants using the integrative plasmids pSETneo::sfbR or pSETneo::sfbR2 respectively (see materials and methods). pSETneo (48) was also introduced into the parental strain as control. No differences on growth were observed between the complemented strains and the control.

Introduction of a copy of sfbR into S. filipinensis ΔsfbR boosted its ability to produce filipin III, almost restoring it to parental strain levels, whereas introduction of sfbR2 into S. filipinensis ΔsfbR2, reduced its ability to produce the antifungal to the same values as the wild type strain (Fig. 3B). These results indicate that both regulators control filipin biosynthesis in opposite ways.

Counteraction between GBL receptor and pseudo-receptor is not uncommon in Streptomyces spp. In S. pristinaespiralis SpbR activates pristinamycin production whereas both two pseudo-receptors PapR3 and PapR5 repress its production (14,49). Similarly, in S. aureofaciens GBL receptor SagR and pseudo-receptor Aur1R behave as auricin biosynthesis activator and repressor respectively (25,50). In S. venezuelae the same type of competition has also been described (7,21).

SfbR and SfbR2 control fil genes expression indirectly.

In order to study if the effect on filipin production in the mutants was a direct consequence of a higher or lower transcription of filipin biosynthetic genes when
compared to the parental strain, we performed gene expression studies by RT-qPCR.

Total RNA was prepared from cultures after growth for 48 h in YEME medium without sucrose, and used for analysis. The transcriptional levels of selected genes corresponding to different operons governing filipin biosynthesis in the mutant strains were compared with those of the wild strain, to which a relative expression value of 1 was assigned. The genes selected included the polyketide synthases *filA1* and *filA2*, the thioesterase encoding *filH*, and two cluster situated regulators (*filR* and *filF*) (26).

In agreement with the overproduction of filipin in the ΔsfbR2 mutant or the decreased filipin production in the ΔsfbR mutant, all the selected genes showed the same pattern of expression, i.e. overexpression in the ΔsfbR2 mutant and repression in the ΔsfbR mutant (Fig. 4), which indicates that SfbR2 is a repressor and SfbR an activator of filipin production.

In the absence of their ligands, GBL receptors recognize and bind to palindromic sequences rich in adenine and thymine called AREs (autoregulatory elements) present in the promoter regions of target genes (in many occasions their own encoding genes), repressing them (14, 20, 51, 52). Recent studies have shown that pseudo-receptors are also capable of recognizing and binding to the same operator sequences (24, 25, 36, 53).

Bioinformatic analysis of the sequence of intergenic regions within the *fil* cluster revealed no ARE sequences, thus suggesting that neither SfbR nor SfbR2 can bind to these regions, and that the control exerted by either regulator must be indirect via a second transcriptional regulator/s.

Contrary to what we have observed in *S. filipinensis*, in most of the cases reported the control of secondary metabolite biosynthesis takes place directly by binding of the
receptor and/or pseudo-receptor to ARE sequences at the promoters of key genes of secondary metabolite gene clusters. Such control has been described for ScbR/ScbR2 which control coelomicin biosynthesis in \textit{S. coelicolor} (54,21), AvaR1/AvaR2/AvaR3 that regulate avermectin production in \textit{S. avermitilis} (23,55,56), SpbR/PapR3/PapR5 controlling pristinamycin biosynthesis in \textit{S. pristinaespiralis} (14,49), or JadR2/JadR3 which control jadomycin production in \textit{S. venezuelae} (7,21), among others. However, a similar case to the \textit{S. filipinensis} paradigm can be found in \textit{S. chatanoogensis} where SprA, a GBL receptor, stimulates transcription of several pimaricin biosynthetic genes and antifungal production in an indirect manner (15).

**Organization of transcriptional units within the GBL cluster**

To obtain an overall picture of the transcriptional arrangement of the \textit{sfb} genes in \textit{S. filipinensis} it was necessary to determine the operons governing their transcription. Because of their divergent localization, the \textit{sfb7} and \textit{sfbR2} genes must have their own promoters. As for the rest of the genes of the group, to analyse the possible coupled transcription of neighbouring genes we performed RT-PCR using RNA from 48 h mycelia. These analyses detected transcripts containing the intergenic regions between \textit{sfb4} and \textit{sfb5} and between \textit{sfb5} and \textit{sfb6} (Fig. S5) thus suggesting that these genes could constitute an operon. Similarly, we detected a transcript containing the intergenic region \textit{sfbR2-sfbA}, whereas no amplification was observed between \textit{sfb1} and \textit{sfb2}, \textit{sfb2} and \textit{sfbR}, or between \textit{sfbR} and \textit{sfb4} (Fig. S5). These results indicate that \textit{sfbA} can be transcribed as part of a bicistronic transcript from the \textit{sfbR2} promoter while \textit{sfb2}, \textit{sfbR} and \textit{sfb4} must have their own promoters (see below) (Fig. 1A).

**Characterization of promoters of the GBL gene cluster containing ARE sequences.**
Analysis of the GBL gene cluster revealed five possible ARE sequences located in the upstream regions of genes sfb2, sfbR, sfb7, sfbR2 and sfbA (Figs. 1A and 5). To assess whether those regions constituted real promoters we determined the transcriptional start points (TSPs) of those genes by 5´-RACE. The corresponding -10 and -35 boxes of each promoter were established by comparison with the matrices reported by Bourn and Babb (57) for Streptomyces that take into account the nucleotides occurring in 13-nucleotide stretches, including the -10 or -35 consensus hexamers (see materials and methods). Results are summarized in Fig. 5.

The sfb2 TSP is located at an adenine 35 nucleotides upstream the ATG start codon. Analysis of the upstream sequence revealed TATCAT and AGTAGT as the -10 and -35 boxes. Both boxes are separated by 14 nucleotides, with the -10 hexamer centred at 10 nucleotides from the TSP (Fig. 5). The TSP of sfbR is located at a thymine 59 nucleotides upstream from the GTG start codon. The sequence TAGCAT, centred at position -9, constitutes the -10 consensus, and a -35 box CCGCCC was identified at 19 nucleotides distance. In the case of sfb7, the TSP was identified at a cytosine 84 nucleotides upstream from the ATG. The -10 and -35 boxes (TTTAAT and TGGTCC, respectively) were centred at positions -9 and -37 nucleotides from the TSP and are separated by 22 nucleotides (Fig. 5). For its part, sfbR2 presented two TSPs, one at an adenine and the second one at a guanine located 234 and 108 nucleotides, respectively, upstream from the ATG start codon. The one at position -234 corresponds to a promoter with -10 (CAGGGT) and -35 (CTGTCC) boxes separated by 19 nucleotides, while the one at position -108 obeys to a promoter with -10 and -35 boxes (TTTGT and CGGAGC, respectively) separated by 21 nucleotides (Fig. 5). Finally, the TSP of sfbA
was identified at a guanine 56 nucleotides upstream from the ATG codon. The analysis of
the upstream sequence revealed a clear promoter, with the -10 box TATATT located 10
nucleotides upstream from the observed TSP and the -35 box TCGCCC separated by 21
nucleotides (Fig. 5). This finding, together with former results indicates that sfbA can be
transcribed as a monocistronic transcript from its own promoter and as a bicistronic
transcript from the sfbR2 promoter.

Interestingly, the putative ARE sequence of sfb2 promoter overlapped the -35 box
while the remaining ARE sequences overlapped -10 boxes of the promoters studied (Fig.
5). The significance of this finding is unclear.

Taken together, these results suggest that S. filipinensis may present a rather more
complex control of GBL genes than other Streptomyces spp. On one side, while two
promoters could direct the transcription of the bicistronic sfbR2-sfbA mRNA, only one of
them (the one that is closer to the translation start) contains an ARE box. This suggests
that these genes could partially avoid self-regulation when being transcribed from the
distal promoter. On the other side, sfbA also has its own dedicated promoter which
contains an ARE box. This feature suggests that sfbA transcription could have various
points of control, either by SfbR or SfbR2, or both.

**Transcription of the γ-butyrolactone gene cluster is controlled by both SfbR and
SfbR2.**

In order to examine the role of SfbR and SfbR2 regulators on the transcription of the
genes whose promoters contained ARE sequences, we measured gene expression in the
mutants by RT-qPCR. Total RNAs obtained from 24-, 48- and 72-hour cultures were
used as a template, and the transcriptional levels of each gene in the different strains were
compared with those of the parental strain, which was assigned a relative expression value of 1.

In order to assess the transcription of the deleted genes primers were designed to generate PCR products near the 5′-end of mRNA. Interestingly, transcription of GBL genes was controlled by both SfbR and SfbR2. The absence of SfbR2 caused an increase in the transcription of all the genes studied including that of its own gene, which indicates that it behaves as a repressor of all these genes, in particular at 24 h. In contrast, the absence of SfbR caused an increase in the transcription of all the genes except for its own transcription, which was reduced (Fig. 6). This result indicates that SfbR is an activator of its own synthesis and a repressor of the remaining genes studied. The self-activation of sfbR transcription was completely unexpected since GBL receptors normally act as repressors of their own synthesis (3,23,36,39).

In GBL regulatory systems, it is common for receptors and pseudo-receptors to repress synthase expression (7,15,24,53,58) so that the GBL accumulates very slowly until it reaches a critical concentration when it binds the receptor and releases it from target promoters. According to our results, S. filipinensis follows such general model in which the GBL receptors and pseudo-receptors act as repressors of the synthase gene (20,39). Although normally these receptors regulate their own synthesis directly by binding to ARE sequences located in their promoters, transcriptional analyses did not allow to confirm such point. For this purpose, we decided to purify both regulators and study their binding capacity to the ARE sequences identified.

GST-SfbR and GST-SfbR2 bind the five ARE-containing promoters of the γ-butyrolactone gene cluster.
To confirm that the promoters containing ARE sequences were the actual targets of SfbR and SfbR2 we performed electrophoretic mobility gel shift assays (EMSA) with GST-SfbR or GST-SfbR2 (Fig. S6) and DNA probes containing the five promoters with ARE sequences. The promoter of sfb4, which lacks an ARE-like sequence, was also used as negative control.

Figure 7 shows the results from EMSAs. To discard the possibility that interactions could be produced by the GST moiety of the fusion proteins, control reactions were performed under the same conditions but using pure GST instead of the fusion protein. This binding was negative in all cases, excluding such possibility. In the cases where retarded bands were observed, the intensity of the band(s) was diminished by the addition of the same unlabelled DNA, suggesting that binding was specific.

As expected, both fusion proteins retarded the sfb2, sfbR, sfb7, sfbR2 and sfbA promoters, while sfb4 promoter was not retarded, indicating that neither of them interact with this region. The presence of multiple retardation bands may indicate various protein/DNA stoichiometries, the cooperative binding of monomers, and/or the binding of dimers as has been proposed for the binding of BulR1, the GBL receptor of S. tsukubaensis, to its targets (36).

**DNase I protection studies reveal that both regulators bind the same sites.**

To determine the precise binding sites of both regulators we carried out DNase I footprinting assays. GST–SfbR or GST-SfbR2 protein (2µM) was tested using 5′-end fluorescein-labelled DNA fragments. All analyses were carried out by triplicate. These analyses revealed that both fusion proteins protected a single site in the promoters containing ARE sequences, and that both bound the same sites (Fig. 8).
Assays with the sfb2p promoter region revealed a 30-nucleotide protection in the coding strand (positions -55 to -84 with respect to the sfb2 translation start site). In the bottom strand the protected sequence was 28 bp long, spanning from position -55 to -82, and both regions were displaced by 2 nucleotides (Fig. 8A).

Footprinting assays of the sfbRp region revealed a 29-nucleotide protection in the coding strand (positions -136 to -164 with respect to the sfbR translation start site). In the complementary strand the protected sequence was 30 bp long, spanning from position -128 to -157. In this case, both protected regions were displaced by 7-8 nucleotides (Fig. 8B).

In the case of the sfb7 promoter, a protected region of 28 nucleotides was observed in the coding strand of sfb7 (positions -100 to -127 from sfb7 translation start codon). In the bottom strand the protected sequence was 30 bp long, at positions -83 to -112 (Fig. 8 C). These protected regions were slightly displaced, i.e. they do overlap 13 nucleotides.

Results of the analysis of the sfbR2p promoter region showed a protected stretch extending for 28 bp of the coding strand. This protected region is located at nucleotide positions -117 to -144 with respect to the sfbR2 translational ATG start site. The protection of the reverse strand was 29-nuclotide long (positions -108 to -136), both regions being displaced by 8-9 nucleotides (Fig. 8D).

In the case of the sfbA promoter, a protected region of 28 nucleotides was observed in the coding strand of sfbA (positions -63 to -90 from sfbA translation start codon), and the same was also observed in the bottom strand (positions -63 and -90). Both protected regions were not displaced (Fig. 8E).
Typically, the protected region in the sense strand of the regulated gene is accompanied by a protection in the complimentary strand, both protected regions being slightly displaced (Fig. 8).

**Information content analysis of the SfbR and SfbR2 operators.**

An information-based model of the binding site was constructed, taking into account the 10 protected regions observed in the footprinting assays. A sequence logo (59) that depicts the binding site is shown in Fig. 8F. This site spans 18 nucleotides and adjusts to the consensus AAACVGNNBNNCSGTTT (where V represents A, C, or G; S is C or G; and B is C, G, or T). It is noteworthy that the binding site displays dyad symmetry and is highly similar to consensus sequences recognized by other GBL receptors and pseudo-receptors from *Streptomyces* sp (23,24).

**SfbR2 is the receptor of antimycin A.**

SfbR2 homologues ScbR2, JadR2 and AvaR2 have been described to bind antibiotics as ligands (21-23). These antibiotics may be either endogenous (21) or exogenous (22). The responses of SfbR2 to different antibiotics that rendered positive results with SfbR2 homologues (chloramphenicol (JadR2); kanamycin (AvaR2)) were analyzed by EMSAs using two DNA targets of SfbR2, the promoter regions of *sfbR* and *sfbA* (Fig. 9). Other exogenous antibiotics tested included tetracycline, other aminoglycosides such as spectinomycin or apramycin, and betalactams such as ampicillin. The endogenous macrolide filipin, and the polyketide-non ribosomal peptide antimycin A commonly produced by *Streptomyces* spp. were also assayed. Dissociation of SfbR2-DNA complexes was not induced by antibiotics chloramphenicol, kanamycin, spectinomycin, apramycin, tetracycline or ampicillin, even at 20 mM concentration (Fig. 9), or filipin.
which could only be tested up to 2 mM concentration given its low solubility (not shown). In contrast, complexes were disrupted by antimycin A at 5 mM concentration (Fig. 9). These results strongly suggest that antimycin A is recognized as ligand by SfbR2, which in response relieves its repression from target promoters. No dissociation of SfbR-DNA complexes was observed in the presence of antimycin A thus indicating that GBL receptor does not bind the antibiotic and suggesting that binding of the pseudo-receptor is specific. Our finding supports the concept that antibiotics may have a signalling function in *Streptomyces* and GBL pseudo-receptors serve as receptors of these signals.

To further support the role of antimycin A as SfbR2 ligand, we performed in vivo assays by testing its effect on the transcription of SfbR2 target genes. *S. filipinensis* was grown for 24 h, added growing concentrations of antimycin A (or ethanol as solvent control) and total RNA was isolated one hour later. Transcript quantity was assessed by RT-qPCR using RNAs obtained as template. The transcriptional levels of each gene in the presence of antimycin A were compared with those in its absence, which was assigned a relative expression value of 1. Interestingly, the addition of 10 µM antimycin A clearly increased transcription of every gene (Fig. 9). Moreover, transcription was further incremented when we increased antimycin A concentration to 50 µM. Taken together, these results clearly indicate that SfbR2 not only binds antimycin A but also responds to it in a concentration dependent manner.

So far, and given that *S. filipinensis* genome is not sequenced, we do not know whether it encodes antimycin A biosynthetic gene cluster. We tried to identify antimycin A in 25-fold concentrated cell culture broth extracts using the same HPLC
method used for the detection of filipins and antimycin A as standard (Sigma) but with no success (see Materials and Methods), thus concluding that this antifungal compound is not produced by *S. filipinensis* under the conditions assayed. Hence, this antibiotic could be considered as an exogenous antibiotic used as a signal to modulate SfbR2 DNA-binding activity. Pseudo-receptors have been described to respond to both endogenous (21) and exogenous (22,23) antibiotics as a way to coordinate antibiotic biosynthesis in the producing organism, and the latter seems to be the case of *S. filipinensis*. Future experimental studies will establish the molecular mechanism involved in this process.

**GBL regulatory model in *S. filipinensis*.**

*Streptomyces* GBLs are important signalling molecules to trigger antibiotic production in a quorum sensing dependent manner. In this work, we have characterized the GBL system from *S. filipinensis*, finding that two key players of this system, the GBL receptor and the pseudo-receptor, counteract each other transcription for the modulation of filipin production. Such control over antifungal production involves an indirect effect on the transcription of filipin biosynthetic genes presumably through a yet unidentified regulator (Fig. 10). In this scenario, the GBL receptor SfbR acts as an activator of filipin biosynthesis, whereas the pseudo-receptor SfbR2 behaves as a repressor. Whether there is a connection between this regulation of filipin biosynthesis and the recently described Pho regulation for this strain (29) remains unknown, and further studies will be required to check such possibility.

The structure of the GBL produced by *S. filipinensis* is currently unknown, but by analogy of the genes found in the GBL gene cluster with other systems characterised, it is likely synthesized by the concerted action of the GBL synthase SfbA, the cytochrome
P450 monooxygenase Sfb4 and the nucleoside-diphosphate-sugar epimerase Sfb5 (3,16,17,38).

As occurs with other GBL systems, the GBL receptor and the pseudo-receptor target the same sites at DNA, which are highly similar to previously identified binding sites from other GBL receptors and pseudo-receptors. Both regulators are self-regulated and repress the transcription of three other genes of the GBL cluster, specifically that of the regulators sfb2 (StrR-family) and sfb7 (SARP-family), and the GBL synthase sfbA. SfbR2 represses its own transcription as expected for a pseudo-receptor, but SfbR, contrary to all the GBL receptors known up to date, activates its own synthesis rather than repressing it (Fig. 10).

Moreover, the pseudo-receptor SfbR2 is able to bind and respond to a presumed exogenous antibiotic, antimycin A, thus extending the number of examples indicating that antibiotics are used in *Streptomyces* species as signals to coordinate antibiotic biosynthesis in the producing organism (21-23).

The intricate regulatory network depicted here should provide important clues to understand the regulatory mechanism governing secondary metabolism.

MATERIALS AND METHODS

Microbial strains and genetic procedures.

*S. filipinensis* DSM 40112 growth and sporulation was achieved as described elsewhere (29). *Escherichia coli* strain DH5α was used as a host for DNA manipulation. *E. coli* BL21 (DE3) was used for expression studies. *E. coli* BW25113 [pIJ790] was used for
gene replacement experiments. *E. coli* ET12567 [pUZ8002] was used as donor in intergeneric conjugations with *S. filipinensis* as described (60). pUC19 (New England Biolabs) was used as the routine cloning vector, pSETneo (Am<sup>R</sup>, Neo<sup>R</sup>, pUC18 replicon, ΦC31 attP (48)) was used for intergeneric conjugations, and pGEX-2T (GE Healthcare) was the vector used to construct expression plasmids. Plasmid DNA preparation, DNA digestion, fragment isolation, and transformation of *E. coli* were performed by standard procedures. The DNA probe used for genomic library (26) screening was obtained by PCR amplification of *S. filipinensis* chromosomal DNA with primers GBR1 and GBR2, designed against the N-terminal end of GBL receptors (Table 1), and sequenced to verify that it corresponded to the conserved helix-turn-helix domain of genuine GBL receptors. Polymerase chain reactions were carried out using Hybrid DNA polymerase as described by the enzyme supplier (EURx). DNA sequencing was accomplished by the dideoxynucleotide chain-termination method using the DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare) with an Applied Biosystems ABI 3130XL DNA genetic analyzer (Foster City, CA., USA).

**Isolation of total RNA and reverse transcription-PCR.**

RNA was extracted as described (61) and transcription was studied as indicated (62). Briefly, we used the SuperScript™ One-Step reverse transcriptase-PCR (RT-PCR) system with Platinum® Taq DNA polymerase (Invitrogen) and 150 ng of total RNA as template. Conditions were as follows: first strand complementary DNA (cDNA) synthesis, 50 °C for 40 min followed by heating at 94 °C for 2 min; amplification, 28 cycles of 94 °C for 40 s, 63-67 °C (depending of the set of primers used) for 30 s, and 72 °C for 30 s. Primers (17-23 mers; Table 1) were designed to detect the possible co-
transcription of neighbouring genes. Negative controls were carried out with each set of primers and Platinum® Taq DNA polymerase in order to confirm the absence of contaminating DNA in the RNA preparations. The identity of each amplified product was corroborated by direct sequencing of the PCR product.

Reverse transcription-quantitative PCR.

Reverse transcription of total RNA was performed on selected samples with 5 µg of RNA and 12.5 ng/µl of random hexamer primer (Invitrogen) using SuperScript™ III reverse transcriptase (Invitrogen) as described (62). Reactions were carried out on three biological replicates with three technical replicates each and appropriate controls were included to verify the absence of gDNA contamination in RNA and primer-dimer formation. Primers (Table 1) were designed to generate PCR products between 56 and 147 bp, near the 5′ end of mRNA. The PCR reactions were initiated by incubating the sample at 95°C for 10 min followed by 40 cycles at 95°C for 15 s, and 64-70°C (depending of the set of primers used) for 34 s. To check the specificity of real-time PCR reactions, a DNA melting curve analysis was performed by holding the sample at 60°C for 60 s followed by slow ramping of the temperature to 95°C. Baseline and threshold values were determined by the StepOnePlus software. C\(\text{t}\) values were normalized with respect to rRNA1 mRNA (encoding 16S rRNA). Relative changes in gene expression were quantified using the Pfaffl method (63) and the REST© software (64). The corresponding real-time PCR efficiency (E) of one cycle in the exponential phase was calculated according to the equation E = 10\(^{-\Delta \text{Ct/slope}}\) (65) using 5-fold dilutions of genomic DNA ranging from 0.013 to 40 ng (n=5 or 6 with three replicates for each dilution) with a coefficient of determination \(R^2 > 0.99.\)
Rapid amplification of cDNA ends.

Transcription start points were identified by using a 5′-RACE system for rapid amplification of complementary DNA (cDNA) ends kit (Invitrogen) following the manufacturer’s instructions (version 2.0) and as described (61), using 5 μg of total RNA for first-strand cDNA synthesis and the gene-specific primers listed in Table 1.

Assessment of filipin and antimycin A production.

Filipin and antimycin A production was assessed after growth at 30 ºC in YEME medium without sucrose. To assay filipin in culture broths, 1 vol of culture was extracted with 1 vol of methanol, and further diluted with methanol to bring the absorbance at 338 nm in the range of 0.1 to 0.4 units. Control solutions of pure filipin III (Sigma) were used as controls. The identity of filipin was confirmed by its UV-visible absorption spectrum (absorption peaks at 356, 338, 320 and 311 nm). Quantitative determination of filipin was performed as previously described (26), using a Mediterranea Sea C18 column (4.6x150 mm, particle size, 3 mm) (Teknokroma). For antimycin A production assessment, 1 vol of culture was extracted with 2 vol of ethyl acetate, and dried by rotary evaporation. The pellet was then suspended in methanol prior to HPLC analysis. The same chromatographic method was used for estimation of antimycin A production at 318 nm. Pure antimycin A (Sigma) was used as standard.

Construction of mutants.

Deletion of *sfbR* from *S. filipinensis* chromosome was made by replacing the wild-type gene with a cassette containing an apramycin selective marker using a PCR based system (66). The plasmid pIJ773 containing the apramycin resistance gene (*aac(3)IV*) and the *oriT* replication origin was used as a template. The mutant was constructed using the...
oligonucleotides SfbR-Red-F and SfbR-Red-R (Table 1) as the forward and reverse primers respectively (the sequence identical to the DNA segment upstream from the start codon of sfbR is in lower case italics and the sequence identical to the segment downstream from the stop codon of sfbR is underlined and in lower case). These two long PCR primers were designed to produce a deletion of sfbR just after its start codon leaving only its stop codon behind. The 3′ sequence of each primer matches the right or left end of the disruption cassette (the sequence is shown uppercase in both primers). The extended resistance cassette was amplified by PCR and *E. coli* BW25113 [pIJ790] bearing cosmid 8H10 was electro-transformed with this cassette. The isolated mutant cosmid was introduced into non-methylating *E. coli* ET12567 containing the RP4 derivative pUZ8002. The mutant cosmid was then transferred to *S. filipinensis* by intergeneric conjugation. Double cross-over exconjugants were screened for their apramycin resistance followed by confirmation by PCR. A similar strategy was used for the deletion of sfbR2, but using the plasmid pIJ778 containing the spectinomycin/streptomycin resistance gene (*aadA*) and the *oriT* as template and the primers SfbR2-Red-F and SfbR2-Red-R (Table 1). In this case, double cross-over exconjugants were screened for their spectinomycin resistance.

**Construction of plasmids for gene complementation.**

In order to complement *sfbR* replacement mutant, a 1261 bp DNA fragment containing the entire *sfbR* gene plus its promoter region was amplified by PCR with primers SfbR16F and SfbR12R (Table 1) using *S. filipinensis* chromosomal DNA as template. The PCR product was cloned into an EcoRV-cut pSETneo (47) to yield pSETneo::sfbR.
Similarly, for S. filipinensis ΔsfbR2 gene complementation, a 1489 bp DNA fragment containing sfbR2 gene plus its promoter was amplified by PCR with primers SfbR2F and SfbR2REcoRI (Table 1). The PCR product was cloned into an EcoRI/EcoRV-cut pSETneo to yield pSETneo::sfbR2.

Construction of plasmids for protein expression.

The SfbR gene was amplified for insertion into the GST expression vector pGEX-2T using PCR. The forward primer used SbRF-GST-F introduced a unique BamHI site at the 5’ end of the gene, while the reverse primer SbR-GST-R carries a EcoRI site 7 nucleotides downstream from the TGA translational stop codon (Table 1). The amplified DNA fragment was digested with BamHI and EcoRI and cloned into the same sites of pGEX-2T to generate pGEX-2T::sfbR. The amplified DNA fragment was sequenced from the expression vector in order to discard any mistakes introduced by the DNA polymerase. Similarly, SfbR2 was amplified using the forward primer SbR2-GST-F and the reverse primer Sb2R-GST-R (Table 1). Cloning of the amplified, and digested DNA fragment into pGEX-2T yielded pGEX-2T::sfbR2.

Expression and purification of GST fusion proteins.

E. coli BL21(DE3) cells containing pGEX-2T::sfbR or pGEX-2T::sfbR2 were grown at 30°C in 100 ml LB medium containing 100 µg/ml of ampicillin to an OD600 of 0.5 and induced by adding isopropyl 1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.1 mM, then growth was continued for an additional 5 h at 22°C. Cells were harvested, resuspended in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3), and lysed by sonication using an ultrasonic processor Sonifier B-12 apparatus (Branson Inc.). The insoluble material was separated by centrifugation, and
the soluble fraction was applied to a GSTrap™ HP (GE Healthcare) column. Proteins were eluted with 10mM reduced glutathione in 50 mM Tris-HCl pH 8.0, and conserved in 20% glycerol at -80°C before use. Protein elution was quantified using Bradford reagent and the presence of the fusion protein was assessed by SDS-PAGE (Fig. S6).

DNA-protein binding assays

DNA binding tests were performed by electrophoretic mobility shift assay (EMSA). The DNA fragments used for EMSA were amplified by PCR using the primers listed in Table 1 and S. filipinensis genomic DNA as template, sequenced to confirm the absence of any mutations, and then labelled at both ends with digoxigenin using the DIG Oligonucleotide 3'-End Labelling Kit, 2nd Generation (Roche Applied Science).

A standard binding reaction contained 0.005 ng/µl labelled DNA probe, 100 mM HEPES pH 7.6, 10 mM KCl, 5 mM MgCl₂, 1 mM DTT, 1% Tween 20, 7.8 mM glutathione, 40 µg/ml poly[d(I-C)], 5% glycerol in 10 µl final volume. Reactions were incubated at 30°C for 10 min and then loaded onto 5% polyacrylamide (29:1) native gels in 0.5x TBE buffer. After electrophoresis, DNA was electroblotted onto a nylon membrane (HyBond-N, Amersham Biosciences) in 0.5x TBE buffer. The DNA was fixed by UV cross-linking, detected with anti-digoxigenin antibodies and developed by chemiluminiscence with the CDP-Star™ reagent (Roche Applied Science).

When EMSAs were performed with the addition of antibiotics, antibiotic was added after 10 min preincubation of the probe with the protein (1 µM) and then further incubated for 10 min before loading onto polyacrylamide gels.

Footprinting assays.
DNase I footprinting assays were performed by the fluorescent labelling procedure as described (67), using GST-SfbR or GST-SfbR2 proteins. The DNA fragments used were the same as those used for EMSA experiments, cloned into pUC19, and amplified by PCR using the universal and reverse primers, one of them labelled with 6-carboxyfluorescein. The PCR products were purified after agarose-gel electrophoresis and DNA concentrations were determined with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

For footprinting, 0.42 pmol of labelled DNA fragment was incubated with GST-SfbR or GST-SfbR2 proteins using the same conditions as for gel shift assays. Lyophilized bovine pancreas DNase I (Roche grade I) was reconstituted in 20 mM Tris HCl pH 7.0, 50 mM NaCl, 100 µg/ml BSA, 1 mM DTT, 10% glycerol to a final concentration of 2.5 x 10^{-3} units/µl. Nuclease digestions were carried out with 7.5 x 10^{-3} units at 30ºC for 1 min and stopped with 180 µl of 40 mM EDTA in 9 mM Tris HCl pH 8.0. After phenol-chloroform purification and ethanol precipitation, samples were loaded in an Applied Biosystems ABI 3130 DNA genetic analyzer (Foster City, CA., USA). Results were analysed with the GeneMarker program (SoftGenetics).

**Bioinformatic analysis.**

For ORF identification and protein annotation we used ORFfinder and BLASTp, respectively, at the NCBI server, and for translation the translate tool at ExPASy. The matrices used to search for regions -35 and -10 were those derived from the alignments of class C and class A promoters of Bourn and Babb (57). To search for a combination of ‘class C–n nucleotides of separation–class A’, we included n columns of null values in the combined matrix. To obtain the logo of the binding sites of the regulators SfbR and
SfbR2, we used the BiPad Server (68). Phylogenetic analyses were performed using the software package MEGA version 6 (69). Distance analysis was performed using Neighbour-Journing method according to the two-parameter model. The robustness was subjected to a bootstrap test with 1000 replicates.

**Accession numbers.**

The sequence of the GBL cluster has been deposited in the GenBank database under the accession number MT017918.

**ACKNOWLEDGEMENTS**

This work was supported by the Spanish Ministerio de Economía, Industria y Competitividad (Grants BIO2013-42983-P and PCIN-2016-190 to JFA). The University of León has defrayed part of the open access publication costs. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. The authors declare that they have no conflict of interest.

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Table 1. Primers used in this study.

| Name               | Sequence (5’→3’)                                                                 |
|--------------------|---------------------------------------------------------------------------------|
| **Probe for genomic library screening**           |                                                                                |
| GBR1               | TGGCKMRRCAGGANCVGCC                                                               |
| GBR2               | GAAGTGGAARTASARSGCBCC                                                            |
| **Construction and verification of mutants**      |                                                                                |
| SfbR-Red-F         | gtgtggggggacccgggtcagggtagggattggtgcgtgATTCCGGGGATCCGTCGACC                   |
| SfbR-Red-R         | gtgtgtatccgcgtcgggtctgtgctgatccggcgactacgtATTTAGGCTGGAGCTGCTTC                |
| SfbR2-Red-F        | ggcacggaacggtgctgggagtcgacgactagctcgcgatccggcagtATTCCGGGGATCCGTCGACC         |
| SfbR2-Red-R        | ggcacgactcgcgtcctggcagaagggtcttcaATTAGGCTGGAGCTGCTTC                          |
| sfbR5F             | CCGCACCCTCTCCAGCACCCG                                                            |
| sfbR11F            | GTACCGCGTCTCATTGAGCC                                                            |
| sfbR12R            | GGTGTGTGTGTAGCCGCC                                                              |
| sfbR19R            | GGTGTGTTCGGTCCCCGTAGGCC                                                        |
| sfbR2F             | GCGTTCAGGGCGAGCGGGG                                                            |
| sfbR2.1F           | GCGGAAAGTCAGCGTGCCC                                                            |
| sfbR2R             | GGCGAACGACAGCGGGTCATGG                                                         |
| sfbR2.1R           | TGGACAGCAGCGAGGAGGG                                                            |
| **Construction of plasmids for genetic complementation** |                                                                                |
| SfbR16F            | CGACGACGAGAAATGGCGGTGG                                                         |
| SfbR12R            | GGTGTGTGTGTAGCCGCC                                                             |
| SfbR2F             | GCGTTCAGGGCGAGCGGGG                                                            |
| SfbR2REcoRI        | GGAATTCGGCGAAGCGAAGGGTGTATGG                                                   |
**Construction of plasmids for protein expression**

| Plasmid     | Sequence                                                                 |
|-------------|---------------------------------------------------------------------------|
| SfbR-GST-F  | TACAGGATCCGTGGCGCAGCAGGAACGGGC                                           |
| SfbR-GST-R  | GGGAATTCGCCGTACTCAACCAGTCTGAC                                            |
| SfbR2-GST-F | TACAGGATCCATGGTCAAGCAGGAACGTGC                                           |
| SfbR2-GST-R | GGGAATTCGCTGCTCTCAGCAGGTTCCC                                             |

**Analysis of co-transcription of sfb genes by RT-PCR**

| Primer Set | Forward/Reverse Sequence | Product Size |
|------------|--------------------------|--------------|
| RT-sfb12-F | GCCGCCCAACCACCTGCTG      | sfb1-sfb2 (436 bp) |
| sfbR2-RACE-2 | GCCAGTGCCTCGACGTCTC     |              |
| SfbR16F   | CGACGACGAGAAATGGCGGTG    | sfb2-sfbR (575 bp) |
| RT-sfb2R-R | GCCGCAGCGTGCAGGATCG     |              |
| RT-sfb4-R  | GCCCGCCTCGAGACGGC       | sfbR-sfb4 (391 bp) |
| RT-sfb4-R  | GCCCGCCTCGAGACGGC       |              |
| RT-sfb45-F | CTGGGACGCGGTTGGAGAGG    | sfb4-sfb5 (657 bp) |
| RT-sfb45-R | GGGTACGTGCGCGGCTG       |              |
| RT-sfb56-F | GCCGCAGGCCAGCAGGAAGG    | sfb5-sfb6 (358 bp) |
| RT-sfb56-R | GCCGCAGGCCAGCAGGAAGG    |              |
| RT-sfbR2A-F | CGGGCGTATCGGAGCAGCAG    | sfbR2-sfbA (488 bp) |
| RT-sfbR2A-R | GCGGTGCTCTTCAGGCTG     |              |

**Analysis of fil and sfb genes expression by RT-qPCR**

| Primer Set | Forward/Reverse Sequence | Product Size |
|------------|--------------------------|--------------|
| qfilA1-F   | CGGCTTCCCTCGACAGCAGCATC | filA1 (114 bp) |
| qfilA1-R   | GCTTCCAGGCAACCTCC       |              |
| qfilA2-F   | CGAGGATCTGTTGGAGGTTGGTC | filA2 (128 bp) |
| qfilA2-R   | GCGGGGCTAGGCTTGGTC     |              |
| Primer  | Sequence                      | Length  |
|---------|-------------------------------|---------|
| qfilR-F | AGACATGGCTCTGGAGTGTG          |         |
| qfilR-R | GTGCCCACCGAAGTGCTC           |         |
| qfilF-F | ATCCAGCAGGCGAACCAG           |         |
| qfilF-R | TTGGAGATATTGACGACCAG         |         |
| qfilH-F | CTCCGCAGCTCTCATTCTTCC        |         |
| qfilH-R | AGGGGCTCCTAGATCTTGTC         |         |
| qsfb2-F | GACCATGCGGATTGCGAC           |         |
| qsfb2-R | TAGCGGACCTGCGAGTC            |         |
| qsfbR-F | CGACGGTTCTTTTGTCTTCC         |         |
| qsfbR-R | ACACCTCCCTACCCCTGACC         |         |
| qsfb7-F | CCCACAGTTCCCTCCACCAG         |         |
| qsfb7-R | AAGTACTCGGGCCTTCTG           |         |
| qsfbR2-F | AGTTGTGGGCGGCGTTCTG         |         |
| qsfbR2-R | TACGGCCACGGGAGATGACAC        |         |
| qsfbA-F | AGGTCTTCCTACCCGGAATG         |         |
| qsfbA-R | TGCTGGTGAAGGAGGTGTGC         |         |
| qrrnA1-F | GACGGCAACCGGAAAGACC         |         |
| qrrnA1-R | TGCGGGACTTAACCCAACATC        |         |

**Rapid Amplification of cDNA ends (RACEs)**

| Primer  | Sequence                      | TSP      |
|---------|-------------------------------|---------|
| sfb2-RACE-1 | GCTTGACGCGCCAGGGACG AAC  |         |
| sfb2-RACE-2 | GCCAGTGCCTCGACGTCGCTC   | sfb2 TSP|
| sfb2-RACE-3 | CGGTGATCTGGCGCGTGCCTGC |         |
| sfbR-RACE-1 | GGGACCAGCGGCAAGGAGGG  | sfbR TSP|
| sfbR-RACE-2   | GTCCACCTTGCCGTGCCCG       |
|---------------|---------------------------|
| sfbR-RACE-3   | TCAGGTAGGCGAGCAGCAGGC     |
| sfb7-RACE-1   | GCGGAAGTCCAGCGTGCCC       |
| sfb7-RACE-2   | GTCTCCAGGAGATAACGCCCGCG   |
| sfb7-RACE-3   | CGCCCCACAGTTCTCCACCC      |
| sfbR2-RACE-1  | CGCACCTCCCCCACACCC       |
| sfbR2-RACE-2  | ACCTCCCCACACCCCGC        |
| sfbR2-RACE-3  | GGCCCATCCGCTTCTCGC       |
| sfbA-RACE-1   | GGACGTGCCCCTATGCCAG       |
| sfbA-RACE-2   | CAGGAAGTGATGCCCCAGCGG     |
| sfbA-RACE-3   | AAGAGACCGACCTGCAGAATGG    |

| EMSAs and footprintings probes |
|-------------------------------|
| sfb2p-EMSA-F  | GCCGCCCAACACCTGCTG         |
| sfb2-RACE-3   | CGGTGATCTGGGCGTGCTGC       |
| sfbRp-EMSA-F  | CCGCACCCGTTTCGAGCGCG       |
| RT-sfb2R-R    | GCCGCAGCGGTCAGGATCG        |
| sfb4p-EMSA-F  | CAGCAGCGGCACGGCAAGG        |
| sfb4p-EMSA-R  | GGGGTCCAACACGAGGTCGCC      |
| sfb7p-EMSA-F  | GCGTGTGCCCAGCCGTCG         |
| sfb7p-EMSA-R  | GCACACCCACCCCGGCACACG      |
| sfbR2p-EMSA-F | AGCTCGTCCCCCTGCCCCG        |
| sfbR2p-EMSA-R | TGGACAGCAAGCGAGGAGGCG      |
| sfbAp-EMSA-F  | GAGAGCAGCCCCCTTCGCAACG     |
Fig 1. GBL gene cluster from *S. filipinensis* and other *Streptomyces*. A) The pointed boxes indicate the direction of transcription. The location of identified ARE sequences is indicated by yellow boxes. B) Genetic organization of GBL clusters in several *Streptomyces* species. The GBL receptor gene is marked in orange and the pseudo-receptor in green. Genes coding for other regulators are shaded in blue, while those genes that could be involved in the GBL biosynthesis are in purple (AfsA-like protein), violet (P450 monooxygenase), pink (dehydrogenase), or brown (Acyl-CoA oxidase). Deduced transcriptional units are indicated by arrows.

Fig 2. SfbR and SfbR2 phylogenetic tree. Homologues used for phylogenetic analyses where chosen randomly among those that were well characterised and belonged to representative *Streptomyces* species. The tree was constructed using the Neihbour-Joining method. NCBI accession numbers are indicated between brackets and calculated pl values are shown between square brackets. Proteins that show atypical pl values are in red. The reliability of each node was analyzed by the bootstrap test with 1000 replicates and the percentage obtained was indicated. Bar indicates 0.1 substitutions per amino acid position.

Fig 3. SfbR inactivation decreases filipin production and SfbR2 deletion increases it.

A) Time course quantification of filipin III production and growth curves in the wild-type
and mutant strains. Fermentations were carried out at 30 °C in YEME medium. B) Effects of gene complementation in YEME medium. Growth curves were identical in all cases. Data are the average of three duplicate flasks. Vertical bars indicate standard deviation of the mean values.

**Fig 4. Gene expression analysis of filipin biosynthetic genes in the mutant strains.** A) Filipin biosynthetic gene cluster. Transcriptional units (26) are indicated by bent arrows. B) Transcription was assessed by RT-qPCR. Total RNA was prepared after growth for 48 h in YEME medium without sucrose. The relative values are referred to 1, the assigned relative value for gene expression in the parental strain. The expression of *rrnA1* (encoding 16S rRNA) was used as control. Error bars were calculated by measuring the standard deviation of the ratio value among three biological and three technical replicates of each sample. Fold change values are indicated below. Primers are listed in Table 1.

**Fig 5. The promoters of some genes of the GBL cluster contain ARE sequences.** The position of the transcriptional start point was determined by 5′ RACE. The putative -10 and -35 hexanucleotides are boxed. Scores resulting from the comparison to the matrices reported by Bourn and Babb (57) for *Streptomyces* are indicated between brackets. The TSP is indicated by a bent arrow and bold type letter. Nucleotides showing homology with the 16S RNA, which could form a ribosome-binding site, are shaded in pink. Putative ARE sequences are shaded in blue. Start codons are highlighted in black.

**Fig 6. Gene expression analysis of *sfb* genes in the mutants.** Transcription was assessed by RT-qPCR. The relative values are referred to 1, the assigned relative value for gene expression in the parental strain. The expression of *rrnA1* (encoding 16S rRNA) was used as control. Error bars were calculated by measuring the standard deviation of
the ratio value among three biological and three technical replicates of each sample. The RNA templates were from 24, 48 and 72 h cultures grown in YEME medium without sucrose. Fold change values are indicated below. Primers are listed in Table 1.

**Fig 7. EMSAs of GST-SfbR and GST-SfbR2 binding to different promoters.**

A) Promoter names are indicated above the pictures. All experiments were carried out with 0.05 ng labelled DNA probe and increasing concentrations of fusion protein (0 to 5 μM).

B) Example of control reactions with pure GST (5 μM) and sfb2p, and competition experiments with 1 μM protein. SC: specific competitor; NSC: non-specific competitor.

**Fig 8. Identification of binding sites.** DNaseI footprints of the GST-SfbR and GST-SfbR2 proteins bound to the different promoter regions. Promoter names are indicated above the pictures. In each panel (A-E), the upper electropherogram (blue line) is the control reaction. The protected nucleotide sequence is shaded in grey. Coordinates are from the translation start point. F) Sequence logo of the nucleotide sequences that constitute SfbR and SfbR2 binding sites. The logo was constructed with the 10 protected regions observed in the footprinting assays. The height of each letter is proportional to the frequency of the base, and the height of the letter stack is the conservation in bits at that position (59). The total information ($R_{\text{sequence}}$) for the binding site is 13.59 bits (0.76 bits per base).

**Fig 9. Antimycin A is SfbR2 ligand.**

A) EMSAs of GST-SfbR2 with various antibiotics. Antibiotics concentration is indicated above the picture. Ampicillin and kanamycin were dissolved in water, while antimycin A and chloramphenicol were resuspended in ethanol, and tetracycline in 0.15 M NaOH. Ethanol or 0.15 M NaOH were used as solvent control when needed. B) Effect of antimycin A on transcription of SfbR2 target genes in vivo.
Antimycin A was dissolved in ethanol and added to 24-h cultures at 10 or 50 µM. Total RNA was isolated one hour later and expression was assessed by RT-qPCR. Transcription of each gene is expressed relative to control value (ethanol added), defined as 1. The expression of *rrnA1* (encoding 16S rRNA) was used as control. Error bars were calculated by measuring the standard deviation of the ratio value among three biological and three technical replicates of each sample. Fold change values are indicated below. Primers are listed in Table 1.

**Fig 10. GBL regulatory model in *S. filipinensis.*** Solid-line arrow/bar: direct activation/repression. Dashed-line arrow/bar: indirect activation/repression. Dotted lines: hypothetical. Lines in brown represent transcription and translation of *sfbR* or *sfbR2*. Gene colouring as in Fig 1.
A

B

S. avermitilis

S. coelicolor

S. fradiae

S. lavendulae

S. pristinaespiralis

S. tsukubaensis
**S. filipinensis ΔsfbR**

| Gene   | 24 h | 48 h | 72 h |
|--------|------|------|------|
| sfb2   | ↑ 58.4 | ↑ 2.4 | ↓ 1.7 |
| sfbR   | ↑ 1.1 | ↓ 16.9 | ↓ 70.9 |
| sfb7   | ↑ 96.6 | ↑ 1.8 | ↑ 3.0 |
| sfbR2  | ↑ 741.4 | ↑ 49.2 | ↑ 63.4 |
| sfbA   | ↑ 31.3 | ↑ 26.1 | ↑ 5.0 |

**S. filipinensis ΔsfbR2**

| Gene   | 24 h | 48 h | 72 h |
|--------|------|------|------|
| sfb2   | ↑ 144.2 | ↑ 20.9 | ↑ 5.6 |
| sfbR   | ↑ 5.9 | ↑ 1.9 | ↑ 1.4 |
| sfb7   | ↑ 97.4 | ↑ 4.8 | ↑ 3.9 |
| sfbR2  | ↑ 322.2 | ↑ 33.6 | ↑ 69.2 |
| sfbA   | ↑ 28.9 | ↑ 31.5 | ↑ 31.3 |
Filipin biosynthesis

Sfb4

Sfb5

SfbA

γ-butyrolactone

SfbR

sfb1

sfb2

sfbR

sfb4

sfb5

sfb6

sfb7

sfbR2

sfbA

Unknown secondary metabolite biosynthesis regulation

Antimycin A

Filipin biosynthesis