

A novel role of 'pseudo' γ -butyrolactone receptors in controlling γ -butyrolactone biosynthesis in *Streptomyces*

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Summary

In streptomycetes, a quorum-sensing mechanism mediated by γ -butyrolactones (GBLs) and their cognate receptors was known to trigger secondary metabolism and morphological differentiation. However, many aspects on the control of GBL signal production are not understood. In this work, we report that ScbR2, the pseudo GBL receptor in *Streptomyces coelicolor*, negatively controls the biosynthesis of γ -butyrolactone (SCB1) by directly repressing the transcription of *scbA*, which encodes the key enzyme for SCB1 biosynthesis. Similarly, the pseudo GBL receptor JadR2 in *Streptomyces venezuelae* was shown to repress the expression of *jadW1*, which also encodes the putative GBL synthase. These regulatory relationships were verified in *Escherichia coli* using *lux*-based reporter constructs. Additionally, the temporal expression profiles of *scbA*, *scbR2* and *scbR* (receptor gene for SCB1) were examined in *Streptomyces coelicolor*, which showed the sequential expression of ScbR/R2 regulators in the control of SCB1 production. Overall, our results clearly demonstrated that pseudo GBL receptors play a novel role in controlling GBL biosynthesis in streptomycetes. As ScbR/R2 homologues and their binding sites upstream of GBL synthase genes are commonly found in *Streptomyces* species, and ScbR2 homologues cross-recognize each other's target promoters, the ScbA/R/R2 quorum-sensing regulatory system appears to represent an evolutionarily conserved signal control mechanism.

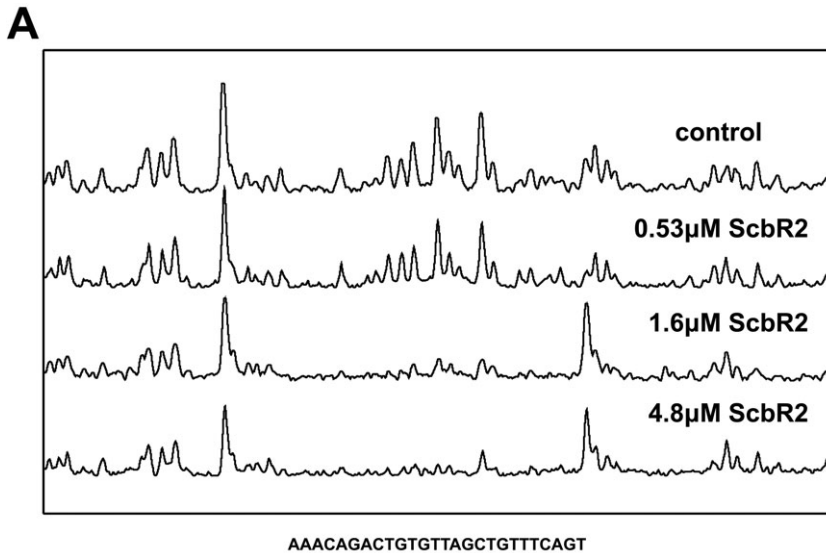
Introduction

The Gram-positive, soil-dwelling, filamentous bacterial genus *Streptomyces* employs γ -butyrolactones (GBLs) as quorum-sensing signals to co-ordinate population behaviours (Bibb, 2005). Although the structures of GBL molecules vary in different *Streptomyces* species, they have a common role in triggering secondary metabolism and/or morphological differentiation (Takano, 2006; Kato *et al.*, 2007). The best studied GBL molecule is A-factor (2-isocapryloyl-3R-hydroxymethyl- γ -butyrolactone) from *S. griseus* (Horinouchi and Beppu, 1994). A-factor is produced in a growth-dependent manner (Horinouchi, 2002), after a peak level, it diminishes rapidly (Ando *et al.*, 1997a). The first dedicated step in the synthesis of A-factor is catalysed by AfsA (Ando *et al.*, 1997b). In the A-factor regulatory cascade, once a threshold concentration is reached, A-factor interacts with its cognate receptor, ArpA, to derepress the expression of AdpA, a key pleiotropic regulator, which initiates a sequence of metabolic and developmental events by activating many target genes (Ohnishi *et al.*, 2005). Despite the significance of GBL-mediated signalling in regulating cellular functions in streptomycetes (Takano *et al.*, 2005; Bunet *et al.*, 2008; Healy *et al.*, 2009), many aspects concerning the control of GBL signals are not yet understood.

For the regulation of GBL signal production, one of the best characterized examples is reported in *S. coelicolor* A3(2), in which the *afsA* homologue, *scbA* was shown to be regulated by the SCB1 receptor protein ScbR, by a complex mechanism (Mehra *et al.*, 2008). SCB1 is the major GBL molecule in *S. coelicolor* (Takano *et al.*, 2000). It is transiently produced at transition to stationary phase, which also coincides with the transcription profile of *scbA* (Takano *et al.*, 2001). Previous investigations indicated that ScbR, the cognate receptor of SCB1, can negatively regulate its own transcription, and that of *scbA* (Takano *et al.*, 2001). Moreover, ScbR is necessary for the induction of *scbA*, which is probably due to the activator role exerted by a ScbA-ScbR complex (Mehra *et al.*, 2008). But till now, how the production of SCB1 signal is controlled is not fully understood.

Recently, with the accumulation of genome data, a phylogenetic analysis of GBL receptor homologues in different streptomycetes became possible, and these proteins

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were reported to diverge into three major branches (Nishida *et al.*, 2007). Members from two distinct branches have been functionally characterized. One branch contains the genuine GBL receptors represented by ScbR; another branch represented by ScbR2 (Xu *et al.*, 2010a) includes proteins designated pseudo GBL receptors, due to their inability to bind GBL molecules.

The coding genes for the pseudo GBL receptors commonly reside in an antibiotic biosynthetic gene cluster, and the typical role of these receptors is to negatively regulate antibiotic production by repressing the transcription of a cluster-situated activator gene (Xu *et al.*, 2010a; Bunet *et al.*, 2011). Interestingly, these receptors bind and respond to multiple endogenous antibiotics, hence, may play an important role in cross-regulation between different antibiotic pathways. During the study of ScbR2 in *S. coelicolor*, we identified its binding site in the promoter region of *kasO*, which encodes an activator of the cryptic type I polyketide synthase (*cpk*) gene cluster. Interestingly, the binding site almost completely overlaps with one of ScbR binding sites in the *kasO* promoter region, which raises the possibility that ScbR2 might also bind other ScbR target sites. In the process of exploring the regulatory relationship between ScbR2 and *scbA*, we discovered a novel role of ScbR2 in repressing *scbA* expression, and hence SCB1 production. We also demonstrated that

Fig. 1. DNase I footprinting of the coding strand of the *kasO* promoter region identified using His₆-ScbR2.

A. The fluorograms correspond to the control DNA (10 μM BSA) and to the protection reactions (with increasing concentrations of 0.53, 1.6 and 4.8 μM of ScbR2 respectively). B. Nucleotide sequences of the promoter regions of *kasO*. The numbers on the left indicate the length of the sequence. The transcriptional start site is indicated by a bent arrow and an asterisk. Sequence protected from DNase I digestion is indicated with shaded boxes. Presumptive -10 and -35 regions of the *kasO* promoter are underlined. The dotted arrows indicate the characteristic inverted repeat sequences, and the dashed lines labelled with Site OA and Site OB indicate the corresponding sites protected by ScbR. The *kasO* translational start codon is marked by a box, and amino acids translated were given below the nucleotide sequences.

another putative pseudo GBL receptor JadR2 in *Streptomyces venezuelae* ISP5230 plays a similar role in controlling GBL biosynthesis. Based on these results, a more complete picture on regulation of the quorum-sensing signal production in streptomycetes has emerged.

Results

Determination of the ScbR2 operator sequences in the *kasO* promoter region

In our previous study, ScbR2 was shown to interact with the *kasO* promoter (Xu *et al.*, 2010a). To determine the specific ScbR2-binding sequences, DNase I footprinting of the *kasO* promoter in presence or absence of ScbR2 was performed, using a capillary sequencer to analyse the protected regions. As seen in Fig. 1 and Fig. S1, the coding strand of the *kasO* showed a main protected region of 25 nt, extending from positions -242 to -218, relative to the transcription start site (TSS). Full protection was observed at 1.6 μM His₆-ScbR2, and this site encompasses a 6 nt inverted repeat (CTGTTT) with 10 bp spacing.

Interestingly, the core of this binding site overlapped completely with that of Site OB (position -222 to -244), which is one of the two ScbR protected sites (OA and OB)

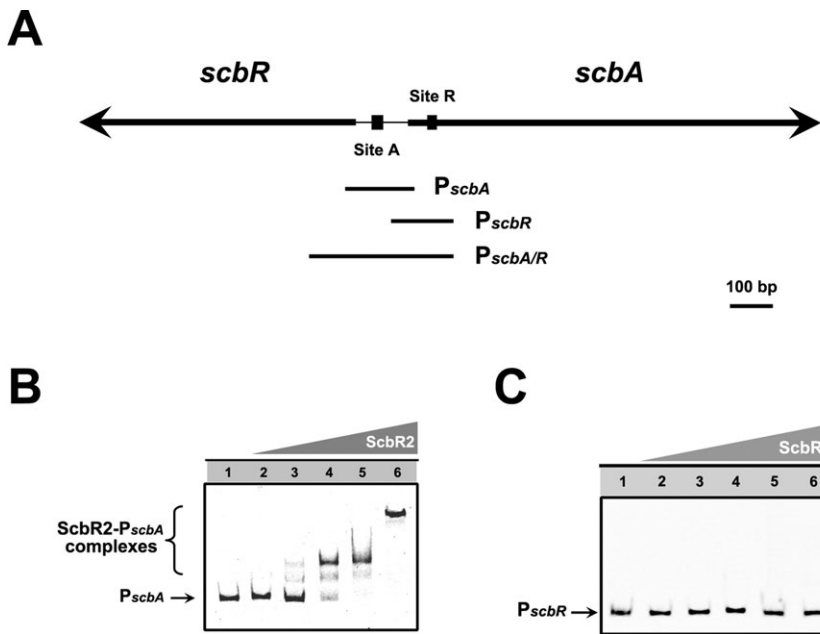


Fig. 2. Binding of ScbR2 to the *scbA/R* intergenic region.

A. A schematic representation of the relative positions of *scbA* and *scbR* in *S. coelicolor* M145. The positions of Site A and Site R are indicated by the rectangular boxes. P_{scbA} and P_{scbR} denote the *scbA* and *scbR* promoter fragments used for gel retardation respectively, and P_{scbA/R} denotes the *scbA/R* promoter region used for DNase I protection experiments. The solid lines indicate the promoter region that it covers.

B and C. Band-shift assays of the interaction of P_{scbA} (B) and P_{scbR} (C) with purified ScbR2 protein. Each lane contained 1 µg probe. Lanes 1–6 contain 0, 12, 24, 60, 120 and 240 nM purified ScbR2 respectively.

in the *kasO* promoter region (Takano *et al.*, 2005) (Fig. 1B). ScbR is also known to bind two sites (A and R) (Fig. 2A) in the *scbA/R* promoter region (Takano *et al.*, 2001). As a consensus sequences could be extracted between Site OB of the *kasO* promoter region and Site A of the *scbA/R* promoter region (position –4 to –33 from the *scbA* transcriptional start site) (Takano *et al.*, 2001), the possibility that ScbR2 also binds the *scbA/R* promoter region is considered. To test this possibility, DNA probes covering the *scbA* and *scbR* promoter regions, respectively, were examined for their ability to interact with purified His₆-ScbR2 in gel mobility shift assays.

ScbR2 interacts with the *scbA* promoter region in vitro and represses *scbA* transcription in vivo

It turned out that ScbR2 binds the promoter region of *scbA* (P_{scbA}) in a concentration-dependent manner (Fig. 2B). Retardation was readily detected upon addition of 1.6 µM ScbR2. This binding is specific as no retardation was observed with the promoter region of *scbR* (P_{scbR}) (Fig. 2C). The results indicate again that ScbR2, unlike ScbR, only recognizes one Site (A) in the *scbA/R* promoter region. To further confirm the binding of ScbR2 with P_{scbA}, DNase I footprinting was performed to locate the DNA binding sites of ScbR2 in the *scbA/R* promoter region. As expected, only one protected region was identified, and it encompasses position +5 to –30 relative to the *scbA* transcriptional start site that partially overlaps with Site A (Fig. 3 and Fig. S2), and this site corresponds to position +50 to +84 relative to the *scbR* transcriptional start site. The full protection of this site requires the same amount of ScbR2 (1.6 µM) as above,

and the protected sequence did not expand when the concentration of ScbR2 was further increased (Fig. 3A). Above results lend further support that ScbR2 specifically binds P_{scbA}, and the core of ScbR2 binding site overlaps with Site A of the *scbA/R* promoter, covering the –10 region of P_{scbA}. It is thus evident that ScbR2 might negatively regulate the transcription of *scbA* by blocking the access of RNA polymerase to its promoter region.

To test the repression of *scbA* transcription by ScbR2, expression profiles of *scbA* in *S. coelicolor* M145 and *scbR2* disruption mutant (ScbR2DM) (Table 1) were analysed by real-time PCR, using total RNAs isolated from mycelia samples harvested at different time points during growth in SMM liquid medium. The expression of the essential sigma factor gene *hrdB* was used as an internal control (Fig. 4A). In M145, a strong transient peak of *scbA* transcript was detected around 36h. Similar to the pattern previously reported (Takano *et al.*, 2001), the transcription of *scbA* was markedly induced at the transition phase and declined quickly upon entry into the stationary phase. In ScbR2DM, while *scbA* expression level at 24 h was similar to that of M145, it became markedly higher (more than 15-fold) after 36 h, and remained so for all subsequent time points. Importantly, the expression level of *scbA* in ScbR2DM did not decline rapidly to negligible level as it did in M145 upon entry into the stationary phase. In fact, it maintained at a relatively high level throughout the stationary phase. Therefore, these results suggest that ScbR2 represses the expression of *scbA* in M145 *in vivo*, and the repression effect is most apparent in the stationary phase. By monitoring the transcriptional profile of *scbR2* in M145 (Fig. S3), we discovered that the

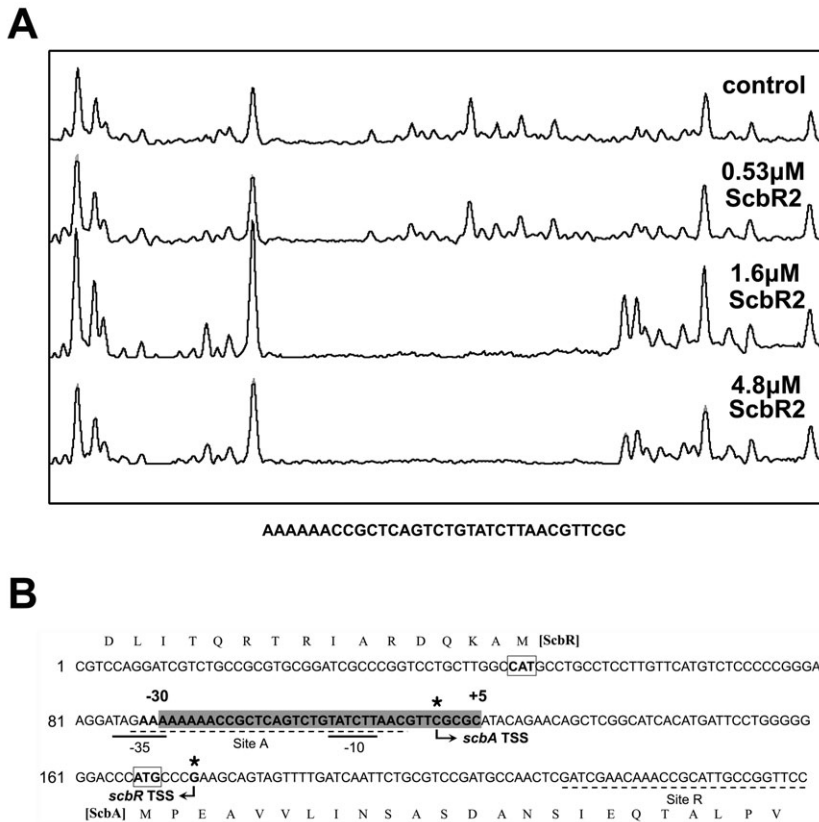


Fig. 3. DNase I footprinting of the coding strand of the *scbA* promoter region using His₆-ScbR2.

A. The fluorograms correspond to the control DNA (10 µM BSA) and to the protection reactions (with increasing concentrations of 0.53, 1.6 and 4.8 µM ScbR2 respectively). B. Nucleotide sequences of the promoter regions of *scbA*. The numbers on the left indicate the length of the sequence. The transcriptional start sites of *scbA* and *scbR* are indicated by bent arrows and asterisks. Presumptive -10 and -35 regions of the *scbA* promoter were underlined. The dashed lines labelled with Site A and Site R indicate the corresponding sites protected by ScbR. Sequence protected from DNase I digestion was indicated with shaded boxes. The *ScbA* and *ScbR* translational start codon were marked by boxes, and the amino acids are translated were given below the nucleotide sequences.

peak of *scbR2* expression also coincided with the rapid drop in *scbA* expression.

The regulatory relationship between ScbR2 and *scbA* was further verified heterologously using an *Escherichia coli*-based reporter system (Tahlan *et al.*, 2007). The *P_{scbA}* promoter used in gel shift assay was cloned upstream of

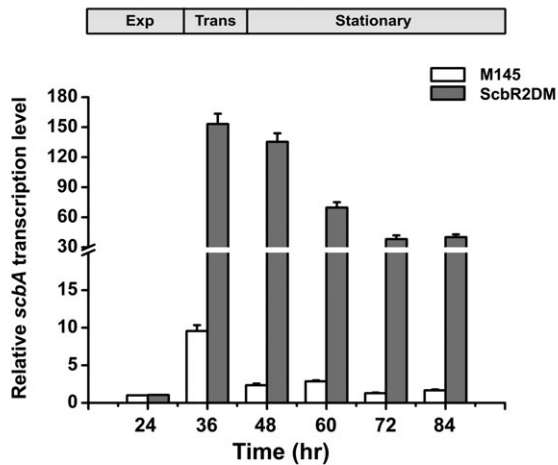
the *lux* operon in the reporter vector pCS26-*Pac* (Table 1). The resulting plasmid pOscbAlux, when introduced into *E. coli*, conferred notable levels of bioluminescence compared with promoterless vector pCS26-*Pac*. Then the control plasmid pACYC184 and previously constructed pScbR2 (Xu *et al.*, 2010a) were introduced into the *E. coli*

Table 1. Strains and plasmids used in this study.

Strains/plasmids	Relevant characteristics	Source/reference
Bacterial strains		
<i>S. coelicolor</i>		
M145	Plasmid-free derivative of <i>S. coelicolor</i> A(3)2	Kieser <i>et al.</i> (2000)
ScbR2DM	The <i>scbR2</i> disruption mutant	Xu <i>et al.</i> (2010a)
ScbR2COM	The complementation strain of ScbR2DM	Xu <i>et al.</i> (2010a)
<i>S. venezuelae</i>		
ISP5230		Yang <i>et al.</i> (1995)
JadR2DM	The <i>jadR2</i> disruption mutant	Yang <i>et al.</i> (1995)
Plasmids		
pET23b-ScbR2	For his ₆ -ScbR2 expression (amp ^R)	Xu <i>et al.</i> (2010a)
pET23b-JadR2	For his ₆ -JadR2 expression (amp ^R)	Xu <i>et al.</i> (2010a)
pCS26- <i>Pac</i>	Promoterless <i>lux</i> reporter (kan ^R)	Tahlan <i>et al.</i> (2007)
pOscbAlux	pCS26- <i>Pac</i> containing the <i>scbA</i> promoter region (kan ^R)	This study
pOjadW1lux	pCS26- <i>Pac</i> containing the <i>jadW1</i> promoter region (kan ^R)	This study
pACYC184	For repressor protein expression in the biosensors (cam ^R , tet ^R)	Tahlan <i>et al.</i> (2007)
pScbR2	For ScbR2 expression in the ScbR2-based biosensor (cam ^R)	Xu <i>et al.</i> (2010a)
pJadR2	For JadR2 expression in the JadR2-based biosensor (cam ^R)	Xu <i>et al.</i> (2010a)

amp^R, ampicillin resistance; kan^R, kanamycin resistance; cam^R, chloramphenicol resistance; tet^R, tetracycline resistance.

A



B

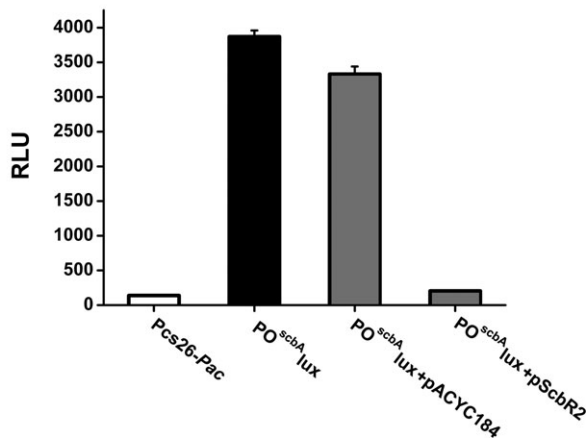


Fig. 4. Analyses of regulatory relationship between ScbR2 and *scbA* in *S. coelicolor* and in *E. coli*.
 A. Quantitative analyses of *scbA* expression profile in *S. coelicolor* M145 and ScbR2DM during different time points in a growth cycle by real-time PCR. Relative values were obtained using *hrdB* as reference. The relative value for *scbA* expression at 24 h in M145 was arbitrarily assigned as one. Note: the levels of *hrdB* transcript at later time points (60, 72 and 84 h) declined from a constant level by less than one half.
 B. Bioluminescence levels detected in *E. coli* containing various plasmid combinations. All values are in relative light units (RLU) and represent the average of at least three independent readings.

strain bearing pOscbAlux respectively, and the resulting transformants were measured for their luciferase activities. It is clear from Fig. 4B that pScbR2 eliminated pOscbAlux-dependent bioluminescence, while the control plasmid pACYC184 had no such effect. These results agree with those obtained from *in vivo* expression profiling in *S. coelicolor*, and provided convincing evidences that ScbR2 directly represses the transcription of *scbA*.

As *scbR* is divergently transcribed from *scbA* (Fig. 2A), and the binding site of ScbR2 in P_{scbA} corresponds to

position +50 to +84 relative to the *scbR* transcriptional start site, the influence of *scbR2* disruption on the transcription of *scbR* was also examined by real-time PCR. As shown in Fig. S4A, the *scbR* expression levels were higher at all time points (except for 24 h) than those of M145, though the degree of increase is lower relative to that of *scbA*. Therefore, it appears that disruption of *scbR2* had negative effect on the expression of *scbR*, but it is uncertain whether this effect is direct. In order to ascertain the regulatory relationship between ScbR2 and *scbR*, we cloned the promoter region of *scbR* (covering both Site A and Site R) into pCS26-Pac, generating pOscbRlux, and assessed the direct effect of ScbR2 on the bioluminescence by introducing pScbR2. As shown in Fig. S4B, compared with the control plasmid pACYC184, pOscbRlux-dependent bioluminescence was not influenced by pScbR2, thus clearly demonstrated that effect of disrupting *scbR2* on transcription of *scbR* is indirect.

Disruption of *scbR2* leads to overproduction of SCB1 in *S. coelicolor*

The ability of ScbR2DM to produce the γ -butyrolactone signal, SCB1, was first assessed by bioassay. To verify that the SCB1 overproducing phenotype of ScbR2DM is due to *scbR2* disruption, the complementation strain of ScbR2DM, i.e. ScbR2COM was also tested for its SCB1 production levels. Culture supernatants from M145, ScbR2DM and ScbR2COM were collected at different time points, extracted with ethyl acetate, and the extractions were subjected to high-performance liquid chromatography (HPLC) to obtain the partially purified SCB1 samples respectively (Xu *et al.*, 2010a). The presence of SCB1 in these samples is verified by electrospray ionization-MS/MS (ESI-MS/MS). The SCB1 fractions were then concentrated and spotted onto confluent lawns of M145. By comparing their abilities to elicit early pigmented antibiotic production, a preliminary test of SCB1 levels in these samples were obtained. The results as presented in Fig. 5 indicate that from 36 to 84 h, SCB1 was constantly produced in ScbR2DM, whereas in M145 as well as ScbR2COM, pigment rings were observed only in the 36 and 48 h samples and they were much smaller than those of the ScbR2DM samples. As the SCB1 levels of ScbR2DM was restored to those of M145 at every time points tested, the results demonstrate that the prolonged and increased production of SCB1 in ScbR2DM is indeed due to *scbR2* disruption. These results again support that *scbR2* represses *scbA* expression, disruption of *scbR2* de-repress *scbA* expression, and hence result in the higher level of SCB1 production.

To obtain quantitative measurements of SCB1 levels in the culture supernatants, the samples from M145 and ScbR2DM were further analysed using LC-ESI-MS/MS.

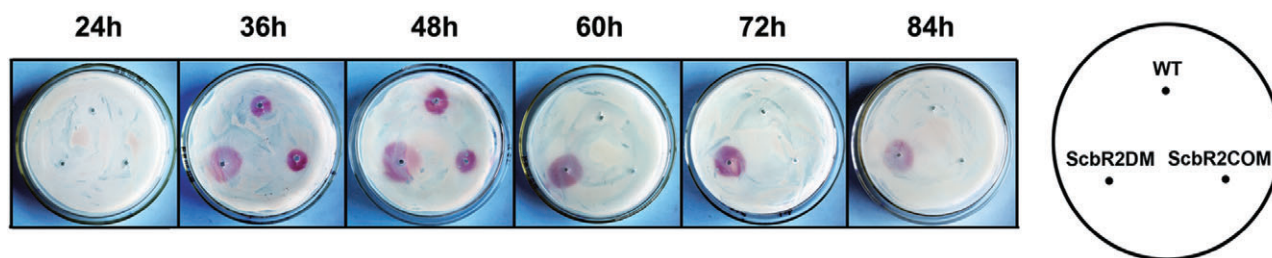


Fig. 5. Bioassays of SCB1 fractions from culture supernatant extracts of *S. coelicolor* M145, ScbR2DM and ScbR2COM. Samples were dissolved in 50% methanol, and spotted on SMMS agar plate that had been spread with a confluent lawn of *S. coelicolor* M145. Plates were incubated at 30°C for 30 h.

According to a previous report (Yang *et al.*, 2005), the characteristic fragment ions of SCB1 are $m/z = 227.0$ $[M + H - H_2O]^+$ and 209.0 $[M + H - 2H_2O]^+$, which were also observed in our ESI-MS/MS analysis (Fig. S5A). Therefore, purified SCB1 was subjected to LC-ESI-MS/MS to monitor the transitions of the protonated molecular ion ($[M + H]^+$) of SCB1 at m/z 245.1→227.0 and m/z 245.1→209.0. As can be deduced from the selected reaction monitoring (SRM) chromatograms in Fig. S5B, the transition peak at m/z 227.0 was more intense than that at m/z 209, therefore it was chosen as the quantifier. Based on the principle that the peak area of the quantifier is proportional to SCB1 concentration in the samples, the ratio of analyte peak area to the respective amount of cell dry weight was calculated for relative levels of SCB1. From Table 2, it is clear that ScbR2DM produced considerably higher levels of SCB1 throughout the growth cycle. For both strains, maximum SCB1 level was detected around 48 h and the level declined afterwards. But the decline is much faster in M145 than ScbR2DM, thereby the most dramatic contrast was observed after the maximal level was reached. This trend agrees well with the quantitative transcriptional profiles of *scbA* *in vivo*, and is also consistent with the results of bioassay experiments. Therefore, the increased transcription of *scbA* detected in ScbR2DM is directly correlated to the

enhancement in SCB1 production, which undoubtedly proves ScbR2 negatively controls SCB1 production.

*JadR2 binds the promoter region of jadW1, which encodes a putative γ -butyrolactone synthase in *S. venezuelae* ISP5230*

To further examine the regulatory role of pseudo GBL receptors, a close homologue of ScbR2, JadR2 in *S. venezuelae* ISP5230 (Yang *et al.*, 1995), was studied. As shown in Fig. 6A, *jadR2* locates adjacent to a gene cluster, designated *jadW1W2W3*. The three genes are transcribed in the same direction, opposite from *jadR2*. Sequence analyses of *jadW1W2W3* suggest they are involved in GBL biosynthesis (Wang and Vining, 2003). *jadW1* encodes the GBL synthase similar to members of the AfsA/BarX family, while *jadW2* encodes homologue of *barS2*, and *jadW3* encodes homologue of *barS1* (the virginiae butanolide biosynthetic genes in *S. virginiae*) (Shikura *et al.*, 2002). Therefore, the three genes are likely involved in GBL biosynthesis in *S. venezuelae*.

To examine the involvement of JadR2 in the regulation of GBL biosynthesis in *S. venezuelae*, purified JadR2 was used in band shift assays with the *jadW1* promoter region (P_{jadW1}) as target. Similar to the relationship between ScbR2 and P_{scbA} , binding of JadR2 with P_{jadW1} was indeed observed. Two retarded bands were visible at different dilutions of the JadR2 protein (Fig. 6B), suggesting that there is only one binding site within the *jadW1* promoter region. As a negative control, the *hrdB* promoter fragment was included, it showed no retardation with an equivalent amount of JadR2 (Fig. S6). These results clearly demonstrate the specific interaction between JadR2 and P_{jadW1} . In subsequent band-shift assays, JadR2 and ScbR2 were shown to bind to each other's target promoters (Fig. S7), indicating that the two regulators are functionally related.

In order to determine the JadR2 binding site in P_{jadW1} , the TSS of *jadW1* was determined by S1 nuclease mapping as described in *Experimental procedures*. Total RNA isolated from 6 h culture of ISP5230 that corresponds to time point of high promoter activity was hybrid-

Table 2. SCB1 levels in *S. coelicolor* M145 and ScbR2DM strains during growth.

Time	ScbR2DM	M145
24 h	0	0
36 h	25 972 ± 2301	2230 ± 308
48 h	50 826 ± 4845	9892 ± 1155
60 h	33 224 ± 2780	965 ± 306
72 h	22 108 ± 1659	492 ± 254
84 h	11 696 ± 1334	0

Mean and SD values calculated from three independent experiments are shown.

SCB1 levels were calculated as the ratio of the analyte peak area of m/z 227.0 to cell dry weight (mg).

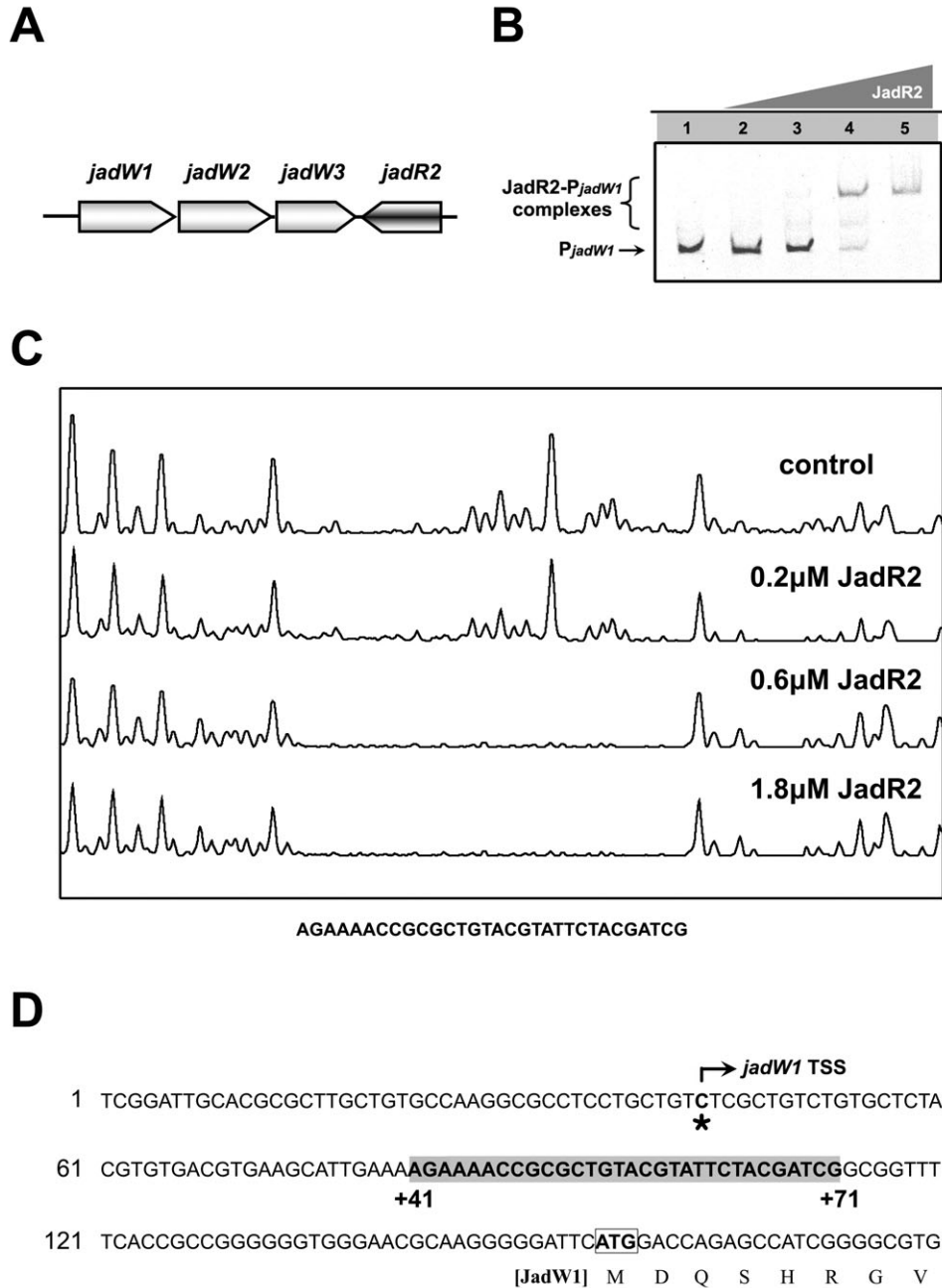


Fig. 6. JadR2 binds the promoter region of *jadW1*.

A. Schematic representation of the relative positions of *jadW1*, *jadW2*, *jadW3* and *jadR2* in *S. venezuelae* ISP5230.

B. Band-shift assay of *P_{jadW1}* with purified JadR2. Each lane contained 1 μg probe. Lanes 1–5 contain 0, 11, 33, 110 and 330 nM purified JadR2 respectively.

C. DNase I footprinting of the coding strand of the *jadW1* promoter region using His₆-JadR2. The fluorograms correspond to the control DNA (10 μM BSA) and to the protection reactions (with increasing concentrations of 0.2, 0.6 and 1.8 μM of JadR2 respectively).

D. Nucleotide sequences of the promoter regions of *jadW1*. The numbers on the left indicate the length of the sequence. The transcriptional start site of *jadW1* is indicated by a bent arrow and an asterisk. Sequence protected from DNase I digestion was indicated with shaded boxes. The *jadW1* translational start codon was marked by a box, and the amino acids translated were given below the nucleotide sequences.

ized with fluorescent-labelled probe (Table S1). The TSS was deduced to be a cytosine 104 nucleotides upstream of the putative *jadW1* start codon (Fig. 6D), from comparison with the respective sequencing reactions (Fig. S8).

DNase I footprinting experiment was subsequently performed to determine the JadR2 binding site in the *P_{jadW1}*. From the fluorograms in Fig. 6C, we can see that only one protected region was detected on the coding strand. The

saturation concentration of JadR2 for stable protein-DNA complex formation is 1.8 μM , and the protected region did not expand even when JadR2 concentration was tripled, reflecting a strong binding sequence specificity. Unexpectedly, the characterized binding site lies downstream of *jadW1* TSS, extending from positions +41 to position +71 in respect to the TSS (Fig. S9). Nevertheless, this situation is similar to a previous report in which the binding site of JadR1 on the *cmlJ* promoter was detected in a region downstream of its TSS, and JadR1 negatively regulates *cmlJ* expression (Xu *et al.*, 2010a).

JadR2 represses the transcription of *jadW1*

To investigate the regulatory relationship between JadR2 and *jadW1*, we first examined the growth curves of *S. venezuelae* ISP5230 and the *jadR2* disruption mutant (JadR2DM) (Table 1) after inoculation into GM medium. The growth patterns of both strains are similar: they both reached stationary phase at around 12 h (Fig. S10). As previous findings indicated that GBL signalling molecules are often abundantly synthesized just before cell growth has ceased, *S. venezuelae* cultures were hence harvested at 2.5, 6, 12 and 24 h time points to investigate *jadW1* transcription in both strains. From the results of real-time PCR (Fig. 7A), it is evident that the expression patterns of *jadW1* are similar to those of *scbA* in *S. coelicolor*, showing a transient peak during 6 and 12 h, and the levels of *jadW1* in JadR2DM exceeded those of ISP5230 in every time points monitored, which undoubtedly showed the negative effect JadR2 had on the expression of *jadW1*. Likewise, the decline of *jadW1* transcription in ISP5230 is much faster than that of JadR2DM, and the most remarkable disparity between the strains was observed in the 12 h to 24 h period.

The repression of *jadW1* expression by JadR2 was also demonstrated in the heterologous host *E. coli* (Fig. 7B). The bioluminescence of pO*jadW1*lux in *E. coli* was reduced after the expression plasmid pJadR2 was introduced, while the control plasmid pACYC184 had no such effect. Therefore, *jadW1* transcription profiling *in vivo* and reporter expression in *E. coli* all suggested that JadR2 in *S. venezuelae*, is involved in control of GBL signal production by directly repressing the transcription of *jadW1*.

Discussion

Pseudo GBL receptors are pivotal repressors in the control of GBL biosynthesis in streptomycetes

Quorum-sensing is a chemical signalling mechanism employed by bacteria to co-ordinate population behaviours. In many Gram-negative bacteria, quorum-sensing is mediated by a LuxR/I system, in which a LuxI-

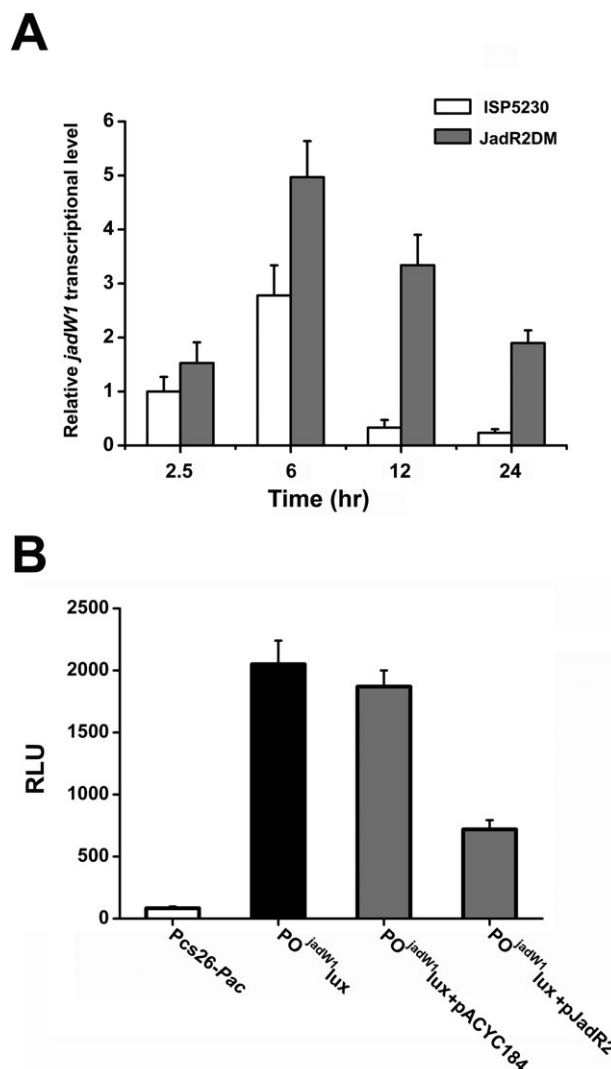


Fig. 7. Analyses of regulatory relationship between JadR2 and *jadW1* in *S. venezuelae* and in *E. coli*.

A. Quantitative analyses of *jadW1* expression profile in *S. venezuelae* ISP5230 and JadR2DM during different time points in a growth cycle by real-time PCR. Relative values were obtained using *hrdB* as reference. The relative value for *jadW1* expression at 2.5 h in ISP5230 was arbitrarily assigned as one.

B. Bioluminescence levels detected in *E. coli* containing various plasmid combinations. All values are in relative light units (RLU) and represent the average of at least three independent readings.

type enzyme produces acylhomoserine lactones (AHLs) signal that is sensed by a cognate LuxR-type response regulator (Fuqua *et al.*, 2001). The direct repression of the LuxI-type signal synthase gene is known to be conducted by RsaL repressors (Rampioni *et al.*, 2006; Mattiuzzo *et al.*, 2011), which constitute a new family of tetrahelical superclass of HTH (helix–turn–helix) DNA-binding proteins (Rampioni *et al.*, 2007). In this study, we revealed the significant effect of *scbR2* and *jadR2* inactivations on the transcription of *scbA* and *jadW1* respectively, highlighting the key role of pseudo GBL receptors as repres-

sors of signal production in streptomycetes. Pseudo GBL receptors, which belong to the TetR-family regulators, are different from RsaL-type repressors in signal sensing and responding properties. Their involvement in quorum-sensing signal control may represent a unique regulatory strategy adopted in streptomycetes, and may serve as a new model to understand signal control mechanism.

However, other regulators or mechanisms might also play a role in the regulation of signal levels. The fact that transcript of *scbA*, production of SCB1 in ScbR2DM and transcript of *jadW1* in JadR2DM still showed a gradual decline after their peaks may reflect the influence of other regulatory mechanisms. For instance in Gram-negative bacteria, besides transcriptional repression, post-transcriptional interference with LuxI-type synthase expression (Pessi *et al.*, 2001) and enzymatic degradation of AHLs (Zhang *et al.*, 2002) were also reported in certain species. Therefore, it is suspected that in streptomycetes, similar mechanisms may also exist to modulate synthase activity or to quickly reduce levels of quorum-sensing signals.

A ScbA/R/R2 quorum-sensing regulatory system in S. coelicolor

As reported previously (Takano *et al.*, 2001), transcription of *scbA* is under significant temporal regulation in *S. coelicolor* M145 (Fig. 4A) to ensure the transient production of SCB1 (Table 2). This transient expression pattern suggests the existence of mechanisms to rapidly turn on and off *scbA* expression. Our study reveals for the first time that pseudo GBL receptor, ScbR2, as a direct repressor of *scbA*, is involved in the turning-off process. Therefore, besides its role in regulating *kasO* expression and mediating antibiotic cross-regulation, ScbR2 plays a novel role in controlling quorum-sensing signal.

In *S. coelicolor*, the production of SCB1 is induced at the late transition phase, under the control of SCB1 receptor protein, ScbR. According to Mehra *et al.* (2008), ScbR represses its own transcription as well as that of *scbA*. Intriguingly, the *scbR* disruption mutants lost the ability to produce SCB1 (Takano *et al.*, 2001), which implies that ScbR may also activate *scbA* transcription (Mehra *et al.*, 2008). Although both ScbR and ScbR2 could regulate *scbA* transcription, they have distinct binding patterns in the *scbA/R* intergenic region: ScbR binds both Site A and Site R, whereas ScbR2 only binds Site A (Fig. 3B). Besides, they have distinct biochemical properties in response to small molecule signals: ScbR is the receptor for SCB1, whereas ScbR2 is the receptor for endogenously produced antibiotics. To provide a full picture of how the two regulators exert their individual influence on controlling the transcriptional of *scbA*, their

expression profiles in M145 obtained by real-time PCR were compared with that of *scbA* (Fig. 8A). *scbA* and *scbR* showed similar transcriptional patterns: from 24 to 48 h, they both rapidly increased and declined; whereas *scbR2* also showed a transient expression pattern in a later time period (from 36 to 72 h). During 36 and 48 h, when the transcript of *scbR2* is rising to the highest level, those of *scbA* and *scbR* are in the process of rapidly decline. In our transcriptional profile analyses, *hrdB* was chosen as the internal control, its expression level declined at later time points (60, 72 and 84 h) from a constant level, which will affect the estimated slope of *scbR2*'s decline.

Integrating the data from previous reports on *scbA/R* regulatory relationship with our findings on the role of ScbR2 in the control of *scbA* expression, a putative regulatory system of ScbA/R/R2 is proposed. As depicted in Fig. 8B, the ligand-free ScbR normally represses its own transcription as well as that of *scbA* during the early exponential growth phase. At that period, ScbR, ScbA and SCB1 are synthesized at basal levels. When the concentration of SCB1 exceeds a threshold, ScbR is released from the operator site of *scbA*, resulting in the de-repression of *scbA* and *scbR*. This may provide the initial induction of *scbA* expression. Further induction of *scbA* expression may involve a co-activator complex formed by ScbA and ScbR (Mehra *et al.*, 2008), which constitutes a positive feed-forward circuit to achieve the rapid induction of *scbA* transcription and SCB1 production. After SCB1 has fulfilled its role in triggering antibiotic production and morphogenesis differentiation, ScbR2 comes into play. Although little is known about how the transcription of *scbR2* is regulated, it is sufficiently induced upon entry into stationary phase to exert its negative effect on *scbA* transcription. With the decrease in *scbA* transcription level, SCB1 biosynthesis is downregulated and the positive feed-forward loop is suspended. Besides, considering the simultaneous repression of *scbA* and *scbR* transcription by ScbR2, it appears that the temporal expression of ScbR2 provide a sufficient mechanism to impair SCB1 production. In all, the inability of ScbR2 to bind SCB1 and its timely expression are the two key factors making it the ideal regulator to repress *scbA* transcription. In a later growth period when antibiotic levels increase, the endogenously produced antibiotics may relieve ScbR2 from its DNA target (Xu *et al.*, 2010a), which perhaps resulted in the slight perturbation of *scbA* transcript after its sharp decline (Fig. 8A). However, more in-depth investigations will be needed to decipher the role of ScbR2 in a complete growth cycle.

It should be noted that regulators other than ScbR and ScbR2 may also be involved in GBL signal control in *S. coelicolor*. For instance, SCO3201 (Xu *et al.*, 2010b)

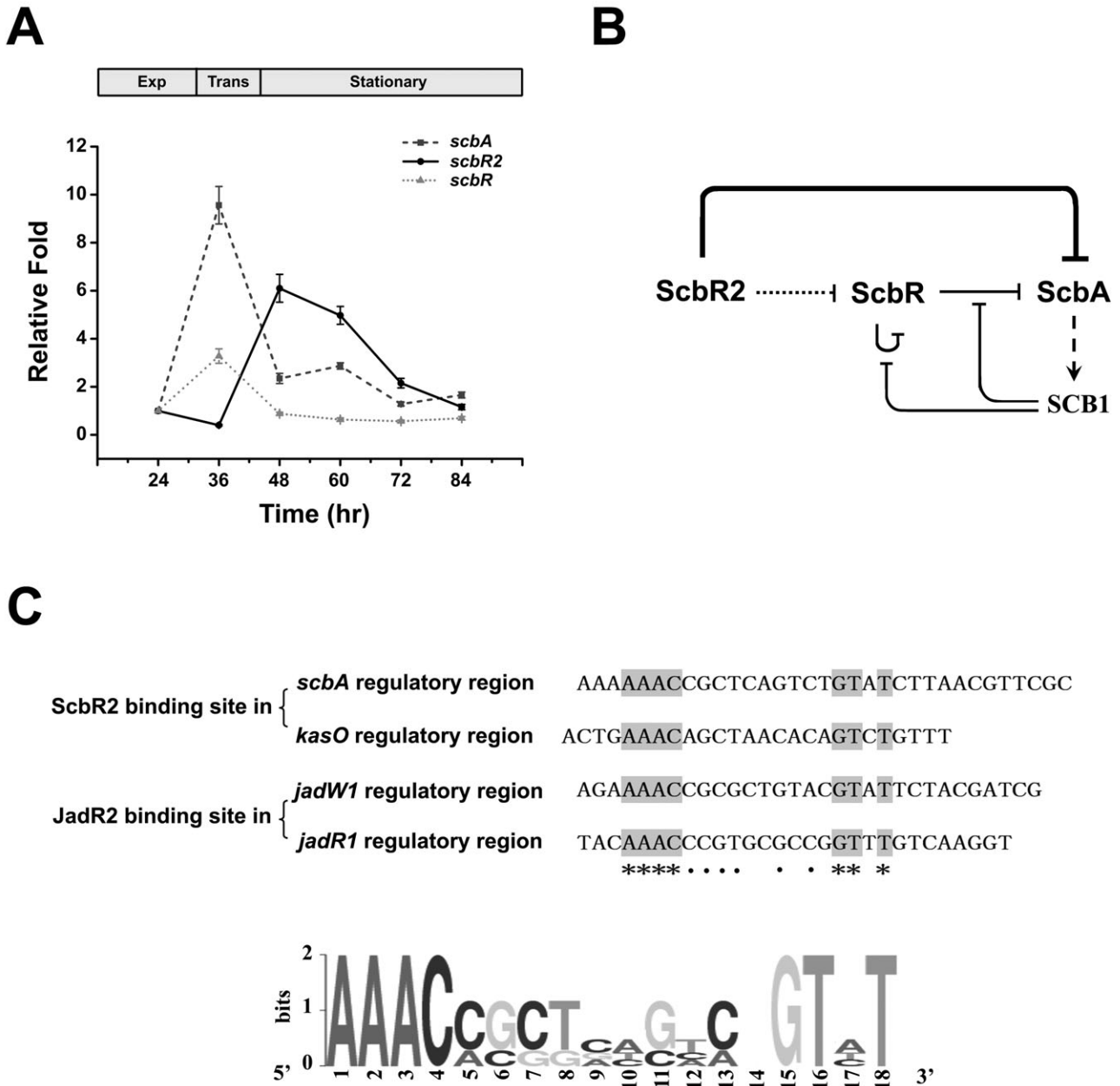


Fig. 8. Expression profiles of *scbA*, *scbR* and *scbR2* in *S. coelicolor* M145.

A. The transcriptional levels of the three genes in *S. coelicolor* M145 were measured by real-time PCR. Quantitative analyses of *scbA*, *scbR* and *scbR2* expressions in *S. coelicolor* M145 at different time points in a growth cycle by real-time PCR. Relative values were obtained using *hrdB* as reference. The relative values for gene expression at 24 h in M145 were arbitrarily assigned as one.

B. Working model for the regulatory roles of ScbR and ScbR2 in control of *scbA* transcription and SCB1 biosynthesis. The production of SCB1 by ScbA is indicated with a dashed arrow. The blunt-ended arrows denote repression of the target gene's transcription, and dotted line means the repression is indirect.

C. Analyses of the consensus sequence of ScbR2 and JadR2 binding sites. Four binding sites were aligned to obtain a consensus sequence. Conserved positions are indicated with asterisks and similar positions are marked by dots. In the sequence logo of ScbR2 and JadR2 binding consensus, the height of each letter is proportional to the frequency of the base appearance.

and NdgR (Yang *et al.*, 2009) were reported to bind the intergenic region between *scbA* and *scbR*, though their exact regulatory functions on GBL signal production remain to be characterized. Interestingly, the global regulator DasR was reported to influence the transcription of

kasO (Rigali *et al.*, 2008). It was predicted that this is also due to direct binding to the *scbA/R* intergenic region (van Wezel and McDowall, 2011). However, DasR may control GBL production in a different mode, by responding to N-acetylglucosamine signal.

Table 3. ScbR/R2 homologues and the predicted binding sites in several *Streptomyces* species.

Strains	ScbR homologues	ScbR2 homologues	AfsA homologues	Predicted binding sites (5'–3')	Positions ^a	References
<i>S. virginiae</i>	BarA	BarB	BarX	GAACCGGTTGGTATGTATCTTA	–178 ~ –156	Matsuno <i>et al.</i> (2004)
<i>S. aureofaciens</i>	SagR	Aur1R	SagA	AAACCGGTTGGTATATATTTT	–176 ~ –196	Novakova <i>et al.</i> (2010)
<i>S. rochei</i>	SrrA	SrrB	SrrX	AAACCGGATAGTATGTATCTTT	–61 ~ –40	Arakawa <i>et al.</i> (2007)
<i>S. lavendulae</i>	FarA	FarR2	FarX	AAACCGGTTGGTATATATTTT	–145 ~ –125	Kitani <i>et al.</i> (2010)
<i>S. acidiscabies</i>	SabR	SabS	SabA	AAACCGGTCGCCTCGTACTCT	+5 ~ +25	Healy <i>et al.</i> (2009)

a. Location in base pair relative to the start codons of the AfsA homologues from GenBank annotations.

ScbR/R2 pairs are widely used in streptomycetes and they share a conserved binding sequence among species

In an earlier report, Nishida *et al.* (2007) conducted a phylogenetic analysis on GBL receptor homologues in streptomycetes, and found that these proteins form several independent branches: among them are the genuine receptor branch represented by ScbR, and the pseudo receptor branch represented by ScbR2. So the phylogenetic tree provides a good indication of functional divergence of these proteins. In this work, several representative *Streptomyces* species in which the GBL synthases had been identified were chosen (Table 3), and the GBL receptor homologues in these strains were subjected to multiple sequence alignment. Likewise, two branches represented by ScbR and ScbR2 respectively were recognized, and the functional divergence is in close agreement with Nishida's report and related literature reports on their functions as genuine or pseudo GBL receptors (Arakawa *et al.*, 2007; Healy *et al.*, 2009; Kitani *et al.*, 2010). Based on these analyses, the corresponding ScbR/R2 homologues in the respective strains are listed in Table 3.

Previously, we have identified the binding sites of JadR2 on the promoter region of *jadR1* (Xu *et al.*, 2010a). In this work, one more binding sites were discovered on the regulatory region of *jadW1*. Along with the two binding sequences identified by ScbR2 footprinting on *scbA* and *kasO* regulatory regions (Fig. 8C), a 18 bp consensus binding sequence was extracted: 5'-AAAC(C/A)(G/C)(C/G)(T/G)(C/G/A)(A/T/C)(G/C)(T/C/A)(C/A)NGT(A/T/C)T-3' (Fig. 8C). Sequence motifs similar to the consensus were searched in the promoter regions of *afsA* homologues using the MEME program (Bailey *et al.*, 2009). The detected binding sequences were listed in Table 3, and they were all bearing high z-score, which indicates high similarity. Besides, in the binding sites of genuine receptor, including ArpA, BarA, FarA and ScbR (Kinoshita *et al.*, 1997; Onaka and Horinouchi, 1997; Takano *et al.*, 2005), highly similar sequences were also detected. These results reflected the strong conservation of the consensus and the general trend that genuine and pseudo GBL

receptors might recognize and regulate similar target genes.

Based on these results, we deduce that these GBL synthase genes are under direct regulation of pseudo GBL receptors, and/or genuine GBL receptors in the respective species. As pairs of ScbR/R2 homologues are commonly found in various streptomycetes, and they share a highly conserved *cis*-element upstream of *afsA* homologues, it appears that the ScbA/R/R2 regulatory system is widely adopted in streptomycetes. Indeed, both *in vitro* (Fig. S7) and *in vivo* (data not shown) binding experiments demonstrated that ScbR2 and JadR2 could recognize each other's target promoters, which lends further support to the evolutionary relationship between these systems.

SCB1 overproduction in ScbR2DM maybe the underlying reason for the changes in antibiotic production levels

Our previous work showed that ScbR2DM, in comparison with M145, was considerably impaired in actinorhodin (Act) and undecylprodigiosin (Red) production (Xu *et al.*, 2010a). But at that time, no reasonable explanation was given to this phenomenon. Here, we propose that these phenotypes are due to the persistence of high levels of SCB1 signals in ScbR2DM. Since the deletion of *scbA*, which abolishes SCB1 biosynthesis, was previously shown to lead to an increase in Act and Red biosynthesis (Takano *et al.*, 2001), it is suggested that GBL systems might be involved in regulating the *act* and *red* clusters at transcriptional level (D'Alia *et al.*, 2011). Moreover, the expression of *cpk* gene cluster in the *scbA* deletion mutant was considerably reduced (D'Alia *et al.*, 2011), indicating that SCB1 signals might positively regulate the expression of *cpk* gene cluster. These speculations would also be consistent with the phenotypes observed for ScbR2DM, in which production of yCPK (the end product of *cpk* cluster) (Gottelt *et al.*, 2010) was considerably upregulated. Taken together, SCB1 overproduction in ScbR2DM may have caused the strong perturbation in the biosynthesis of the three pigmented antibiotics. Therefore,

in rational genetic modifications, pseudo GBL receptors could be a useful target for the optimized production of GBL-dependent antibiotics biosynthetic gene clusters.

Experimental procedures

Bacterial strains and growth conditions

Bacteria strains used in this study are listed in Table 1. *Streptomyces coelicolor* strains M145, ScbR2DM and ScbR2COM were handled as previously described (Xu *et al.*, 2010a). MS agar was used to grow the strains for making spore suspensions. For RNA isolation and SCB1 production, the strains were cultivated in SMM liquid medium (Takano *et al.*, 2005) at 28°C, 250 r.p.m. SMMS agar was used for SCB1 bioassays (Takano *et al.*, 2001). *S. venezuelae* ISP5230 and JadR2DM were grown on MYM agar (Yang *et al.*, 1995) for making spore suspensions. To prepare vegetative inoculums, strains were cultivated in MYM at 28°C, 220 r.p.m. for 20 h. Then the inoculums were transferred into glucose-MOPS (GM) medium (Jakeman *et al.*, 2006) at 4% (v/v). *E. coli* DH5 α was used as a host strain for cloning experiment and luciferase assay. *E. coli* BL21 (DE3) was used as a host strain to express His₆-ScbR2 and His₆-JadR2. They were grown in Luria–Bertani (LB) medium containing ampicillin (100 $\mu\text{g ml}^{-1}$), kanamycin (50 $\mu\text{g ml}^{-1}$) or chloramphenicol (50 $\mu\text{g ml}^{-1}$) when necessary.

Gel mobility shift assays

His₆-tagged ScbR2 and JadR2 were purified from *E. coli* BL21 (DE3) harbouring pET23b::*scbR2* and pET23b::*jadR2*, as described by Xu *et al.* (2010a). For ScbR2-binding experiments, the promoter regions of *scbA* and *scbR* were obtained by PCR from the genomic DNA of *S. coelicolor* M145, using primer pairs PscbAF/PscbAR and PscbRF/PscbRR (Table S1) respectively. For JadR2-binding experiments, the promoter regions of *jadW1* and *hrdB* were amplified by PCR from the genomic DNA of *S. venezuelae* ISP5230, using primer pairs PjadW1F/PjadW1R and PhrdBF/PhrdBR (Table S1) respectively. The subsequent binding experiments were performed using a modified gel mobility shift assay described previously (Li *et al.*, 2010). The DNA probe (1 μg) was incubated with varying concentrations of purified ScbR2 or JadR2 at 25°C for 30 min in a buffer containing 20 mM Tris-base (pH 7.5), 2 mM dithiothreitol (DTT), 5 mM MgCl₂, 0.5 $\mu\text{g ml}^{-1}$ calf BSA and 5% (v/v) glycerol in a total volume of 20 μl . After incubation and electrophoresis, the non-denaturing 4% (w/v) polyacrylamide gels were stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen) for 30 min in TBE (89 mM Tris-base, 89 mM boric acid, 1 mM EDTA, pH 8.0) buffer, and photographed under ultraviolet transillumination using Bio-Rad GelDoc XR.

DNase I footprinting assays

DNase I footprinting assays were performed according to the fluorescent labelling procedure (Zianni *et al.*, 2006). Briefly, DNA fragments were prepared by PCR using fluorescent-

labelled primers listed in Table S1: FAM-kasOF and kasOR for the ScbR2-binding site in the *kasO* promoter, scbAF and HEX-scbAR for ScbR2-binding site in the *scbA* promoter; FAM-jadW1F and jadW1R for the JadR2-binding site in the *jadW1* promoter. After being purified from agarose gel, labelled DNA fragments (120 ng) and respective concentrations of proteins were added to a final reaction volume of 50 μl , and incubated at 25°C for 20 min. DNase I digestions were carried out for 1 min at 25°C and stopped with stop buffer provided by Promega. After phenol-chloroform extraction and ethanol precipitation, the samples were loaded in an Applied Biosystems 3730 DNA genetic analyser together with the internal-lane size standard ROX-500 (Applied Biosystems). The dye primer-based sequencing kit (Thermo) was used to further precisely determine the sequences after aligning the capillary electrophoresis results of reactions (Zianni *et al.*, 2006). Electrophoregrams were then analysed with the GeneMarker v1.8 (Applied Biosystems).

S1 nuclease mapping analysis

Total RNA for S1 nuclease mapping was isolated from *S. venezuelae* ISP5230 after being incubated in GM medium for 6 h. The hybridization probe for *jadW1* was prepared by PCR from *S. venezuelae* ISP5230 genomic DNA using 5'-fluorescein-labelled primer FAM-jadW1R and an unlabelled primer jadW1F (Table S1). For each S1 nuclease reaction, 40 μg of RNA was used for hybridization with 5'-fluorescein-labelled probe in NaTCA buffer at 45°C for 15 h, following denaturation at 65°C for 15 min (Wang *et al.*, 2009). S1 nuclease (Promega) digestions were performed as described by Kieser *et al.* (2000), and the reaction products were loaded in an ABI 3730 DNA analyser along with the molecular size marker ROX-500. Electrophoregrams were then analysed with the GeneMarker v1.8 (Sola-Landa *et al.*, 2005).

RNA isolation and real-time PCR

For *S. coelicolor* strains, culture samples were collected after 24, 36, 48, 60, 72 and 84 h of incubation at 28°C in SMM liquid medium. For *S. venezuelae* strains, samples were collected at 2.5, 6, 12 and 24 h after incubation in GM medium. Total RNA was isolated as described (He *et al.*, 2010). They were subsequently treated with RNase-free DNase (Promega) and checked by PCR to eliminate possible chromosomal DNA contamination.

First strand cDNA synthesis was carried out using 2 μg of each RNA samples and SuperScript III (Invitrogen), following the manufacturer's instructions. Real-time PCR of selected genes were performed using the ABI 7500 Real-Time PCR Detection System and SYBR Green PCR Master Mix (Applied Biosystems). Ten per cent of the cDNA synthesis reaction mixture was used as a template for each subsequent PCR, using the following gene-specific primer pairs: qscbAF/qscbAR and qchrdBF/qchrdBR for detection of *scbA* and *hrdB* transcription in *S. coelicolor* strains respectively, while qjadW1F/qjadW1R and qvhrdBF/qvhrdBR for detection of *jadW1* and *hrdB* transcription in *S. venezuelae* strains respectively. Real-time PCR parameters were set as follows:

95°C for 10 min, followed by 40 two-step amplification cycles consisting of 30 s denaturation at 95°C and 60 s of annealing and extension at 60°C. The results were analysed by ABI 7500 software v2.0.1, and the relative expression levels of target genes were normalized internally to *hrdB* level. Relative transcript levels were quantified by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and shown as relative fold of change in comparison with the 24 h samples of the wild-type strain for *S. coelicolor* and 2.5 h samples of the wild-type strain for *S. venezuelae*. All samples run in triplicate.

Construction of biosensor strains and luciferase assay

The plasmids used in this study are listed in Table 1. The *scbA* and *jadW1* promoter regions used in gel mobility shift assays were ligated to the BamHI-digested and blunt-ended pCS26-*Pac* giving rise to pOscbAlux and pOjadW1lux respectively. The promoter region of *scbR* (covering both Site A and Site R) was obtained by PCR from the genomic DNA of *S. coelicolor* M145, using primer pairs ScbRF/ScbRR (Table S1). It was ligated to the BamHI-digested and blunt-ended pCS26-*Pac*, giving rise to pOscbRlux. Two expression vectors, pScbR2 and pJadR2 were previously constructed (Xu *et al.*, 2010a). After 12 h incubation, bioluminescence of *E. coli* reporter cultures was measured using a 20/20n single tube luminometer (Turner Biosystems).

SCB1 bioassay and LC-MS analysis

To assay for the production of SCB1, the culture supernatants of *S. coelicolor* M145, ScbR2DM and ScbR2COM were extracted with the same volume of ethyl acetate. The ethyl acetate extracts were then evaporated and resuspended in methanol. For SCB1 bioassay sample preparation, the extracts were first partially purified on HPLC to obtain the fraction portion containing SCB1 as described previously (Xu *et al.*, 2010a). Then they were concentrated to the same volume and dissolve in 50% methanol for subsequent SCB1 bioassay (Takano *et al.*, 2001). For quantitative detection of SCB1 levels in the culture supernatant, the extracted samples were directly subjected to HPLC-ESI-MS/MS analysis. Separation was accomplished on Agilent ZORBAX SB-C18 analytical column of 210 mm × 150 mm, using a gradient of H₂O (A) /acetonitrile (B). The linear gradient was from 35% (v/v) B to 75% (v/v) B over 40 min at a flow rate of 0.2 ml/min. Detection was carried out on Thermo Electron LCQ Deca XP ion-trap MS equipped with an electrospray interface (ESI). MS detection was conducted in positive ion mode and data acquisition was performed in SRM mode. The optimized MS parameters were: ion spray voltage was 1.75 kV, capillary temperature was 275°C, and the collision energy was set at 35% for MS/MS.

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