Molecular Microbiology (2005) **56**(2), 465–479 doi:10.1111/j.1365-2958.2005.04543.x

A bacterial hormone (the SCB1) directly controls the expression of a pathway-specific regulatory gene in the cryptic type I polyketide biosynthetic gene cluster of *Streptomyces coelicolor*

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Summary

Gamma-butyrolactone signalling molecules are produced by many *Streptomyces* species, and several have been shown to regulate antibiotic production. In *Streptomyces coelicolor* A3(2) at least one γ-butyrolactone (SCB1) has been shown to stimulate antibiotic production, and genes encoding proteins that are involved in its synthesis (*scbA*) and binding (*scbR*) have been characterized. Expression of these genes is autoregulated by a complex mechanism involving the γ-butyrolactone. In this study, additional genes influenced by ScbR were identified by DNA microarray analysis, and included a cryptic cluster of genes for a hypothetical type I polyketide. Further analysis of this gene cluster revealed that the pathway-specific regulatory gene, *kasO*, is a direct target for regulation by ScbR. Gel retardation and DNase I footprinting analyses identified two potential binding sites for ScbR, one at –3 to –35 nt and the other at –222 to –244 nt upstream of the *kasO* transcriptional start site. Addition of SCB1 eliminated the DNA binding activity of ScbR at both sites. The expression of *kasO* was growth phase regulated in the parent (maximal during transition phase), undetectable in a *scbA* null mutant, and constitutively expressed in a *scbR* null mutant. Addition of SCB1 to the *scbA* mutant restored the expression of *kasO*, indicating that ScbR represses *kasO* until transition phase, when presumably SCB1 accumulates in sufficient quantity to relieve *kasO* repression. Expression of the cryptic antibiotic gene cluster was undetectable in a *kasO* deletion mutant. This is the first report with comprehensive *in vivo* and *in vitro* data to show that a γ-butyrolactone-binding protein directly regulates a secondary metabolite pathway-specific regulatory gene in *Streptomyces*.

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Introduction

Streptomycetes are morphologically complex Gram-positive mycelial soil bacteria that produce a variety of secondary metabolites. Many of these metabolites have antibiotic activity, and some are of great commercial value. They are typically products of intricate biosynthetic pathways that are activated in a growth phase-dependent manner, so that production coincides with the onset of aerial mycelium formation in agar-grown cultures and with stationary phase in liquid (Champness, 2000). This expression pattern is often mediated by pathway-specific regulatory genes in the biosynthetic clusters (Takano *et al*., 1992; Gramajo *et al*., 1993). Many streptomycetes also produce small γ-butyrolactone signalling molecules that play important regulatory roles in the onset of antibiotic production and, sometimes, of morphological differentiation (see review Horinouchi, 2002). The first γ-butyrolactone discovered, and the best characterized, is A-factor (2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone), required in nanomolar concentrations for both antibiotic (streptomycin) production and sporulation in *Streptomyces griseus* (Khoklov *et al*., 1967; Mori, 1983). Other structurally determined γ-butyrolactones include the virginiae butanolides (VB), which control virginiamycin (VM) production in *Streptomyces virginiae* (Yamada *et al*., 1987; Kondo *et al*., 1989), and IM-2, which elicits production of showdomycin and minimycin in *Streptomyces lavendulae* FRI-5 (Sato *et al*., 1989). *Streptomy-*

ces coelicolor A3(2), genetically the most characterized streptomycete, also produces several γ-butyrolactones (Anisova *et al*., 1984; Kawabuchi *et al*., 1997). Recent studies have identified γ-butyrolactones with actinorhodin (Act) and undecylprodigiosin (Red) stimulatory activity in *S. coelicolor*, and the structure of one of these molecules, SCB1, was determined (Takano *et al*., 2000).

In *S. griseus*, A-factor accumulates to detectable levels in the culture medium before the onset of streptomycin production and binds to a cytoplasmic A-factor-binding protein, ArpA (Onaka *et al*., 1995). In the absence of A-factor, ArpA binds to the promoter region of a pleiotropic regulatory gene, *adpA*, repressing its transcription; and as AdpA directly activates transcription of the pathway-specific activator gene for streptomycin biosynthesis, streptomycin is not produced. In the presence of A-factor, ArpA binds to A-factor and no longer represses *adpA* transcription, and streptomycin biosynthesis occurs (Ohnishi *et al*., 1999).

Similar regulatory cascades involving a γ-butyrolactone and its cognate-binding protein have been reported in several other streptomycetes. *S. virginiae* has two *arpA* homologues, *barA* and *barB* (Okamoto *et al*., 1995; Kinoshita *et al*., 1997). BarA (which shows higher similarity to ArpA than does BarB) represses expression of *barB*, and binding of VB to BarA relieves this repression (Kinoshita *et al*., 1997). The exact role of BarB is not known, although it is thought to be part of the BarA regulon that regulates virginiamycin production (Matsuno *et al*., 2004).

The tylosin biosynthesis gene cluster of *S. fradiae* contains five putative regulatory genes. Two of these, *tylP* (highest similarity to *arpA*) and *tylQ* (highest similarity to *barB*), encode γ-butyrolactone-binding protein homologues, while two others, *tylT* and *tylS*, encode homologues of SARPs (*Streptomyces* antibiotic regulatory proteins; Wietzorrek and Bibb, 1997; Bate *et al*., 1999). From mutagenesis studies and promoter expression analysis, these genes are thought to form a regulon with *tylP* at the top of the cascade repressing *tylS* and *tylQ*, whose expression is presumptively activated by an as yet unidentified γ-butyrolactone(s) (Stratigopoulos *et al*., 2002). TylQ represses *tylR*, which encodes an activator for tylosin production (Stratigopoulos and Cundliffe, 2002). *tylS* also seems to regulate *tylR* by activation (Bate *et al*., 2002).

In *Streptomyces pristinaespiralis*, the product of an *arpA* homologue, *spbR*, represses pristinamycin production by binding to the promoter of *papR1*, which encodes a SARP homologue. The γ-butyrolactone presumed to activate production of pristinamycin has yet to be identified (Folcher *et al*., 2001).

Adjacent, diverging genes involved in γ-butyrolactone synthesis (*scbA*) and γ-butyrolactone binding (*scbR*) were isolated from *S. coelicolor* by Takano *et al*. (2001). ScbR regulates transcription of both *scbA* and itself by binding to the divergent promoter region, and the γ-butyrolactone SCB1 inhibits this binding. Furthermore, from *in vivo* analysis, ScbR was found to repress its own expression while activating the expression of *scbA*. An *scbA* mutant overproduced two quite different antibiotics (Act and Red – see above) and was defective in the production of γ-butyrolactones with antibiotic stimulatory activity. The *scbR* mutant was delayed in Red production (Takano *et al*., 2001). Surprisingly, these phenotypes were the opposite of those expected from the A-factor model in *S. griseus*.

The results presented here used DNA microarray analysis as a guide to identify another direct target of *ScbR*, and thus further extended the knowledge of the γ-butyrolactone regulatory cascade in *S. coelicolor*. The new target is part of a cryptic polyketide gene cluster, first identified by Kuczek *et al*. (1997), that lies very close to *scbA* and *scbR* on the chromosome (Bentley *et al*., 2002). ScbR was shown to interact directly with the promoter region of *kasO*, which encodes a SARP homologue, and binding was prevented by the addition of SCB1. Furthermore, a *kasO* deletion mutant was constructed, and the expression of some genes in the cryptic polyketide gene cluster was analysed by reverse transcription polymerase chain reaction (RT-PCR). The expression of these genes was markedly reduced or undetectable in the mutant compared with the parent strain, suggesting that *kasO* is indeed an pathway-specific regulatory gene.

Results

Microarray analysis reveals several genes that are repressed (directly or indirectly) by ScbR

ScbR is an autorepressor, as shown by several observations: transcription from the *scbR* promoter is highly elevated at early exponential phase in an *scbR* null mutant (M752); ScbR binds to the *scbR* promoter; and the addition of exogenous SCB1 γ-butyrolactone to M751 (Δ*scbA*) induces the expression of *scbR* dramatically, and prevents binding of ScbR to the *scbR* promoter region (Takano *et al*., 2001). To identify other genes that might be repressed by ScbR, we used newly available glass slide microarrays spotted with PCR products corresponding to 7333 of the 7825 annotated genes identified by genome sequencing of the M145 strain of *S. coelicolor* A3(2) (Bentley *et al*., 2002; Chater *et al*., 2002). RNA was isolated from replicate cultures of M145 and its *scbR*-deleted derivative M752 at OD_{450} 0.5 (14.0 h) and 0.46 (14.0 h) from growth curve No. 1 (GC1), and OD_{450} 0.38 (15.5 h) and 0.37 (15.5 h) from growth curve No. 2 (GC2), respectively, in supplemented liquid minimal medium (SMM). Both strains grew at the same rate. RNA was also isolated from replicate mid-exponential phase cultures of

Table 1. Gene whose transcripts were found to be more abundant in the *scbR* mutant (M752) than in the parent (M145) (a) and after the addition of SCB1 to the *scbA* mutant (M751) (b). Primary array data can be found in the supplement.

Gene No. (cosmid-based gene No.)[a]	Ratio[b]	*P*-value[c]	Description[a]
(a) Genes overexpressed in the *scbR* mutant (M752)			
SCO6277 (SC1G7.03)	20.2	0.0402	Epoxide hydrolase
SCO6283 (SC1G7.09)	9.45	0.0341	Conserved hypothetical protein
SCO6278 (SC1G7.04)	6.72	0.0464	Integral membrane transport protein
SCO6281 (SC1G7.07)	3.75	0.0212	FAD-binding protein
SCO6284 (SC1G7.10)	3.05	0.0763	Putative decarboxylase
SCO6269 (SCAH10.34c)	2.83	0.00961	*orfY* oxidoreductase beta-subunit
SCO6279 (SC1G7.05)	2.79	0.0231	Diaminobutyrate-pyruvate aminotransferase
SCO4646 (SCD82.17)	2.64	0.0197	*secE* preprotein translocase SecE subunit
SCO1244 (2SCG1.19)	2.34	0.0246	*bioB* biotin synthase
SCO6272 (SC2C4.02)	2.21	0.0427	Secreted FAD-binding protein
SCO5225 (SC7E4.22c)	1.87	0.00364	*nrdM* ribonucleotide-diphosphate reductase small chain
SCO3767 (SCH63.14)	1.74	0.00392	Hypothetical protein
SCO4253 (SCD8A.26c)	1.52	0.00941	Unknown
SCO4703 (SCD31.28)	1.50	0.0137	*rplD* 50S ribisomal protein L4
SCO3534 (SCE2.15)	1.46	0.0130	Large ATP-binding protein
(b) Genes overexpressed in M751 (Δ*scbA*) upon addition of SCB1			
SCO6281 (SC1G7.07)	15.5	0.0169	FAD-binding protein
SCO6278 (SC1G7.04)	14.6	0.0117	Integral membrane transport protein
SCO6283 (SC1G7.09)	13.4	0.0136	Conserved hypothetical protein
SCO6277 (SC1G7.03)	12.8	0.0164	Epoxide hydrolase
SCO6286 (SC1G7.12c)	7.40	0.0512	Regulatory gene
SCO6282 (SC1G7.04)	7.35	0.0240	Oxidoreductase
SCO6273 (SC2C4.03)	5.23	0.0491	Putative type I polyketide synthase
SCO6275 (SC1G7.01c)	4.75	0.0525	Putative type I polyketide synthase
SCO6272 (SC2C4.02)	4.61	0.0488	Secreted FAD-binding protein
SCO6265 (SCAH10.31)	4.38	0.0484	scbR γ-butyrolactone-binding protein
SCO6276 (SC1G7.02)	4.25	0.0488	Secreted protein
SCO6267 (SCAH10.32)	3.99	0.0622	Hypothetical protein (orfA)
SCO1244 (2SCG1.19)	3.15	0.00728	*bioB* biotin synthase

a. The SCO numbers are taken from the EMBL Accession No. AL645882. Cosmid-based gene numbers are also given in brackets. The description of each gene is based on the genome annotation in AL645882.
b. Normalized average ratio of signals from M752 versus M145 for time point 1 (a) and from M751 time zero versus 60 min after addition of SCB1 for (b).
c. *t*-test *P*-value calculated from replicate data.

an *scbA*-deleted derivative of M145 (M751) immediately before (time 0) and 60 min after the addition of chemically synthesized SCB1 (final concentration of 31 ng ml^{-1} = 128 μM). For the microarray experiment with the *scbR* mutant, gene expression in the mutant was compared with that of the wild type at early exponential phase, as genes regulated by ScbR are strongly repressed at this stage in the wild type. Data analysis using GeneSpring (Silicon Genetics) revealed 15 genes with higher expression in M752 than in M145 and 13 genes with higher expression in M751 after addition of SCB1 (Table 1). The genes included *scbR* in the SCB1 addition experiment, as predicted from our previous experiments. The general reliability of the data and the quality of the RNA were supported by S1 nuclease protection analysis of the growth stage-specific genes, *scbR* and *scbA* and of a constitutively expressed gene, *hrdB*, that encodes the major sigma factor of *S. coelicolor* (data not shown). The microarray data on the expression of three of the genes listed in Table 1 were also analysed by

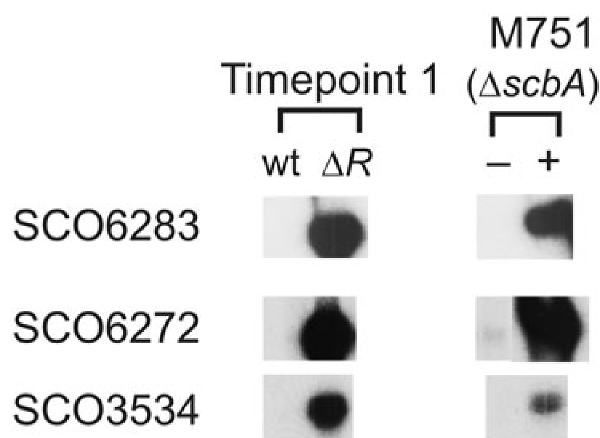

Fig. 1. S1 nuclease protection analysis of three genes identified by the microarray analysis to be overexpressed in the *scbR* mutant. Transcription of SCO6283, SCO6272 and SCO3534 was analysed by S1 nuclease protection experiments using RNA isolated from M145 and *scbR* GC1 at time point 1, OD_{450} 0.36 and 0.39 (M145: 15.5 h, M752: 15.5 h), respectively, and also RNA isolated from Δ*scbA* at OD_{450} 0.41 just before (–) and 60 min after addition of SCB1 (+).

S1 nuclease protection analysis (Fig. 1). Transcripts of all three genes (SCO6283, SCO6272 and SCO3534) were confirmed to be dramatically more abundant in the *scbR* mutant than in the parent strain and all three were strongly induced when SCB1 was added to the *scbA* mutant. Primary array data can be found in the supplement.

The expression of genes that could be involved in Act or Red regulation or biosynthesis was not observably increased at the time point studied in this DNA microarray analysis. This resulted from the *scbR* mutant antibiotic production phenotype: it is repressed in Act production, and Red production had not yet occurred at the time when the RNA was isolated.

None of the genes identified by microarray analysis to be overexpressed in a scbR *mutant appear to be directly regulated by ScbR*

Previously, we showed that ScbR in a crude cell-free extract from an *Escherichia coli*-based expression system could form DNA–protein complexes with the *scbA/scbR* promoter region, as detected by gel retardation (Takano *et al.*, 2001). To test whether ScbR directly regulates the genes identified by microarray analysis, similar gel retardation experiments were conducted using Dig-labelled PCR products corresponding to the regions upstream of most of these genes (Table 2). Genes with coding start sites overlapping the preceding genes (SCO6273, 6277 and 6278) were omitted from the gel retardation analysis as it is likely that these genes are co-transcribed with the gene upstream. In no case was there evidence of the formation of DNA–protein complexes (data not shown), suggesting that the sequences upstream of these genes were not direct targets for ScbR binding, although it could not be excluded that other accessory factors might be needed for binding in any particular case.

In the absence of SCB1, ScbR binds to the promoter region of the possible pathway-specific regulatory gene kasO *of the polyketide cluster containing most of the ScbR-regulated genes*

Many genes identified by the transcriptome analysis are located in a cryptic polyketide gene cluster (Kuczek *et al.*, 1997; K. Pawlik *et al.*, in preparation) (Fig. 2). The structure of the end product of this biosynthetic pathway has not yet been determined, but the sequence indicates the involvement of a multimodular type I

Table 2. List of primers used to create PCR fragments for gel retardation experiments.

Primers	Primer sequence 5′–3′[a]	Template	Fragment size[b] (bp)	Amplified promoter region[c]
accA1F	cagggcgaacgcctcatcagc	SC2C4	504	SCO6271/6272 (*accA1/scF*)
accA1R	gacaccgcggcgcctttcatg			
ksEF	ctcgatcgagtggaccgtctc	SC1G7	502	SCO6282/6283
ksER	ggatcgccgtcgttctggacg			
G119F	gcagaacccggccagttcgct	Total DNA	510	SCO1244
G119R	caggtcgtcgtcggaagtgac			
ksRF	**ctgcta**atggccgtactgacc	Total DNA	268	SCO6279
ksRR	gcagaaacggctcggcgaagc			
ksFF	**tagcta**ctgcgcctccacaag	Total DNA	268	SCO6281
ksFR	cgacggcaccgtgtctgatga			
secEF	gatcatccggtgcacaccaag	SCD82	333	SCO4646 (*secE*)
secER	ccttcttgcctcgcttgccgc			
orfYF	**acgtcat**gtcctcgagccggta	Total DNA	115	SCO6269 (*orfY*)
orfYR	agtccttcatggactgcttgc			
ksSF	gacagtcccacgacagcgatc	SC1G7	502	SCO6275/6276
ksSR	gtgacgttcgcgaaggtctcg			
ksTF	cgctcaccaccgaggagttca	SC1G7	425	SCO6284
ksTR	catggtcacgggcagggaacc			
ksNF	acttctatctcgaccagagcg	SC1G7	372	SCO6288
ksNR	cgagtgtctggcagaccttgg			
R2Fw2	gtcgaactcgggcgtcagctc	SC1G7	430	SCO6286/6287
R2Rev	cctcggcggcggagagaat			
F	gtgtatgtcacggacgaggag	SC1G7	522	SCO6280 (*kasO*)
R	gttggcctgcaacagcaggta			
A1	gcaaggactgctcgccggaca	SC1G7	259	SCO6280 (*kasO*)
A2	ctcagggtcaccgacgctccg			
3	ccctccggagcgtcggtgacc	SC1G7	201	SCO6280 (*kasO*)
4	ggaggccgcgctgcgcgacgt			

a. 5′ to 3′ primer sequences. Bold represent tails added to the original sequence.
b. Size of PCR amplified product.
c. Description of the amplified promoter region. The SCO numbers are taken from the EMBL Accession No. AL645882. Gene names are also given in brackets. In some cases where two genes are divergent to each other, both gene names are given.

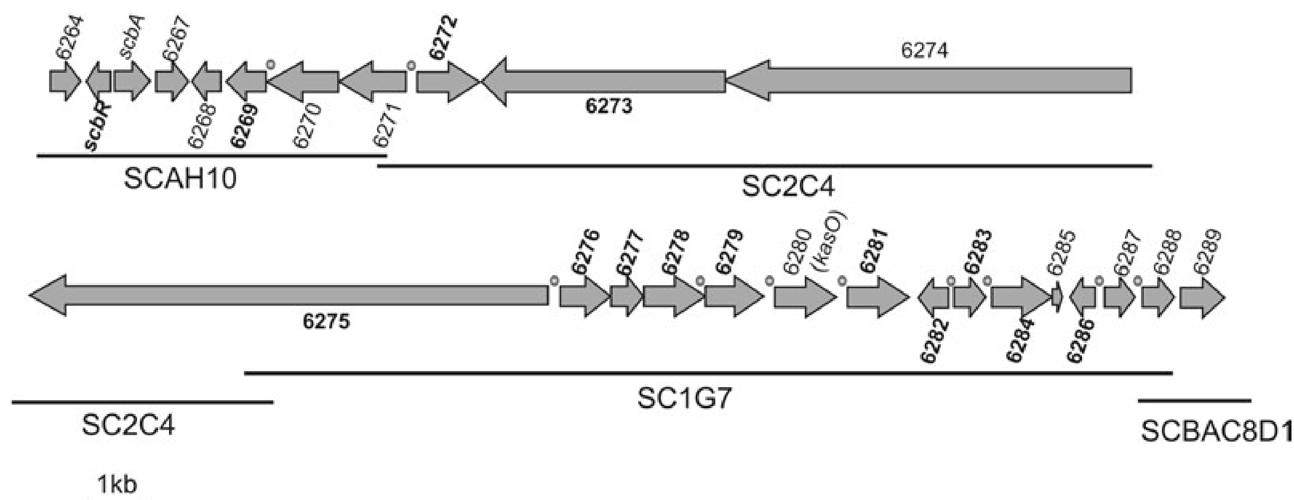

Fig. 2. Genetic organization of DNA near *scbA* and *scbR* (SCO6264–SCO6289). Genes are represented by shaded arrows. Numbers denote SCO gene numbers from the *S. coelicolor* genome sequence (EMBL Accession No. AL645882). Genes identified to be induced in the *scbR* mutant by microarray analysis are indicated in bold. The promoter regions analysed for binding to ScbR by gel retardation experiments are indicated in shaded circles. The genes represented by the cosmids are underlined, with the cosmid numbers indicated below.

polyketide synthase. The fact that we could not obtain evidence of direct binding of ScbR to the regions upstream of these genes (which include the promoter region of one putative regulatory gene SCO6286) suggested that the direct target of ScbR might be one or both of the two other putative regulatory genes present in the polyketide gene cluster: SCO6280 (*kasO*) and SCO6288, which encode homologues of SARPs (Wietzorrek and Bibb, 1997). The expression level of SCO6288 was too low for reliable microarray analysis, and no data were obtained for the other putative pathway-specific regulatory gene, *kasO*, because a PCR probe for this gene was not available on the microarray. Gel retardation experiments were conducted to assess whether ScbR could directly regulate these two regulatory genes.

A DNA–protein complex was observed with the *kasO* promoter fragment (fragment F+R) (Fig. 3A and B), but not with the SCO6288 promoter fragment (data not shown). We also failed to detect complexes with the promoters of SCO6271 and SCO6287, putative biosynthetic genes that had not been highlighted in the microarray analysis. Three retarded *kasO* promoter bands were visible at different dilutions of the ScbR protein, suggesting a possibility of two binding sites within the DNA used for the gel retardation experiment. The *kasO* promoter fragment showed no retardation with an equivalent concentration of ScbR-free extract isolated from *E. coli* JM101. Addition of 100- to 10-fold excess of unlabelled specific PCR product reduced the proportion of the labelled promoter-containing fragment that was fully retarded. No competition was apparent when 100-fold excess unlabelled non-specific *Streptomyces* DNA (the vector plasmid pIJ922) was added. These results indicated a specific interaction between ScbR and the *kasO* promoter region.

In previous work (Takano *et al.*, 2001), we showed that SCB1, the γ-butyrolactone produced by *S. coelicolor*, could prevent binding of ScbR to the *scbA* and *scbR* promoters. Based on this observation, SCB1 and each of its three chemically synthesized stereoisomers were added to the gel retardation assays. Formation of the DNA–protein complex was reduced upon addition of 1 μg (1.6 μM) of SCB1, whereas the addition of equivalent amounts of each stereoisomer of SCB1 had little or no effect (Fig. 3C). In the case of VB type CD+, the effect was very similar to SCB1 in Fig. 3C; however, with repeated experiments the level of relief was less than obtained with the same amount of SCB1 (data not shown). This indicated that ScbR has a high level of specificity for its cognate γ-butyrolactone, and that SCB1 prevents DNA binding activity by ScbR.

In confirmation of the presence of two ScbR binding sites in the *kasO* promoter region, two smaller DNA fragments, kasOA1+A2 (259 bp) and kas3+4 (201 bp), both showed ScbR binding in gel retardation experiments (Fig. 3D). No competition was apparent when 100-fold excess unlabelled pIJ922 (non-specific competitor) was added, indicating that the interactions between ScbR and the two *kasO* promoter regions were specific. Addition of 1 μg (1.6 μM) of SCB1 reduced DNA–protein complex formation in both promoter regions, slightly more effectively with the kasOA1+A2 fragment, suggesting that SCB1 prevents DNA binding by ScbR, in agreement with previous results (Takano *et al.*, 2001).

Fig. 3. Binding of ScbR to the *kasO* promoter region.
A. *kasO* promoter fragments used for gel retardation and DNase I protection experiments. The name of the primers are in the order of the positions of the primers. The arrow (*kasO*p) denotes the potential transcriptional start site of *kasO*. The dotted line denotes the ScbR binding sites explained in more detail in Fig. 5C.
B. Gel retardation experiments with crude extract of *E. coli* JM101 harbouring *scbR*, various controls. The numbers denote the various amounts (μl) of 8× diluted JM101/*scbR* crude extract used. One microlitre of concentrated (8× that of ScbR) *E. coli* JM101 crude extract was used for the negative control.
C. Gel retardation experiments using crude extract of JM101 harbouring *scbR* (2.5 μl of 8× diluted) and supplemented with SCB1 or one of its three stereoisomers. CD (Circular Dichroism) denotes the optical rotation (+ or –) of each γ-butyrolactone.
D. Gel retardation experiment using smaller DNA fragments containing either of the two ScbR binding sites with various controls. 2.5 μl of 8× diluted *E. coli* JM101 harbouring *scbR* was used for each reaction. Quantities used in controls were as in Fig. 4B.

The ScbR binding sites identified by DNase I footprinting analysis

The putative transcriptional start site of *kasO* was determined by high resolution S1 nuclease protection analysis to be 35 nt upstream of the *kasO* translational start site (Fig. 4A). The putative –10 and –35 regions of the *kasO* promoter resemble the consensus sequence for the major and essential sigma factor HrdB (Brown *et al*., 1992).

DNase I footprinting was used to locate the DNA binding sites of ScbR in the *kasO* promoter region. Two protected regions were identified (Fig. 4B). Site OA lies at –3 nt to –34 nt from the *kasO* transcriptional start site and site OB at –222 nt to –244 nt (Fig. 4C). Site OA overlaps the –10 and –35 region of the *kasO* promoter and

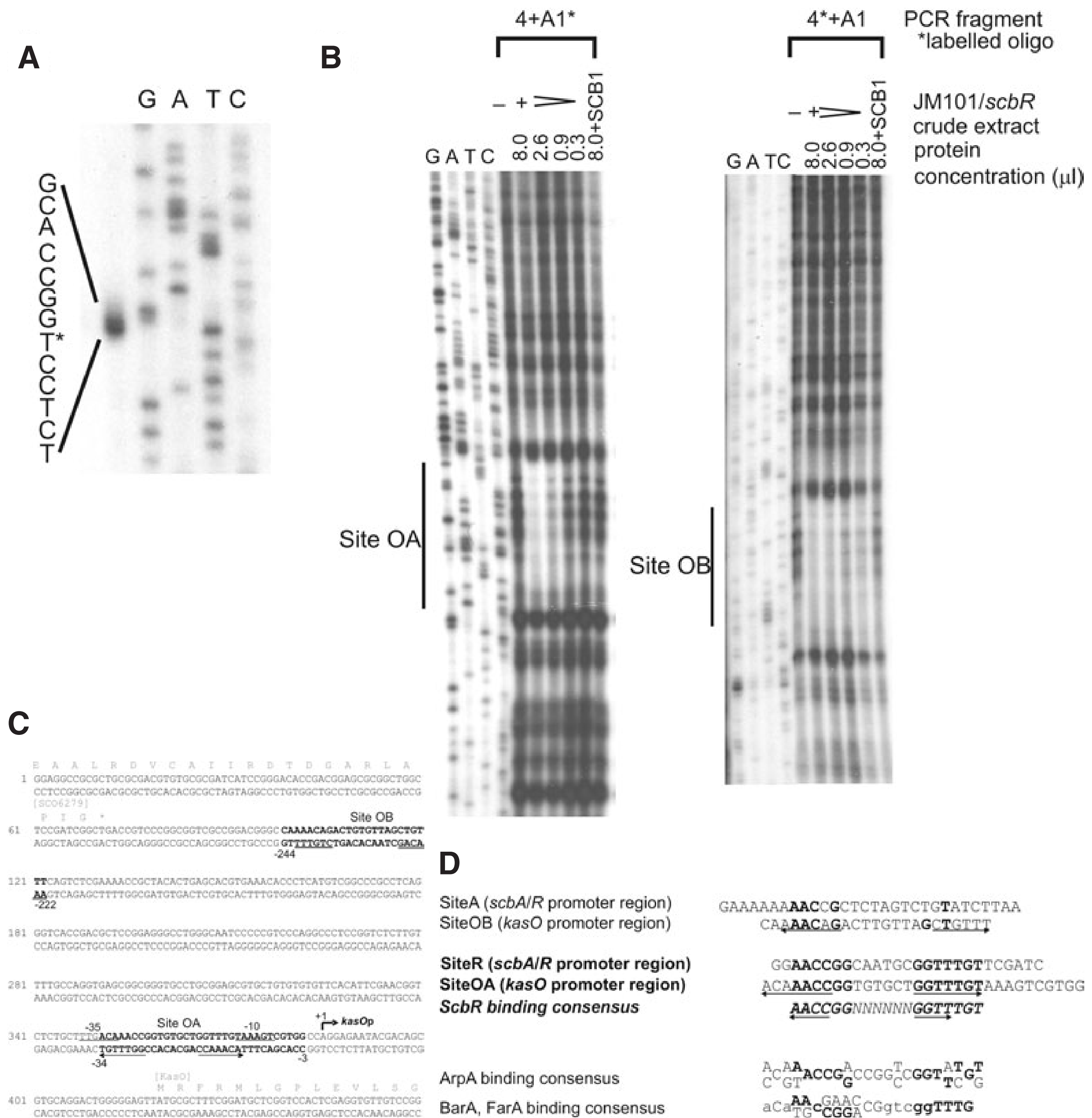

Fig. 4. S1 nuclease transcript mapping and DNase I protection analysis of the *kasO* promoter region.
A. High resolution S1 nuclease protection analysis of the transcriptional start site of *kasO.* An asterisk indicates the probable start site; the sequences shown are those of the template strand. Lanes G, A, T and C are sequence ladders derived from the same labelled primers that were used to generate the PCR products.
B. DNase I protection analysis. The regions that are protected from cleavage by DNase I are shown by vertical lines. Site A covers the –10 and –35 regions of the *kasO* promoter, whereas site B lies further upstream from site A. Opposite DNA strands were assessed for protection by separately labelling each of the oligonucleotides used to generate the PCR product. G, A, T and C ladders were used to locate precisely the protected regions.
C. Sequence of the *kasO* promoter region showing the ScbR binding sites. Sequences protected by ScbR from DNase I cleavage are indicated in bold; numbering is with respect to the *kasO* transcriptional start site, which is shown by an arrow. Presumptive –10 and –35 regions of the *kasO* promoter are underlined. An inverted repeat sequence is shown with underscored arrows. A somewhat similar sequence in site OB (but with different spacing) is also underlined.
D. Consensus ScbR binding sequence. Four ScbR binding sites are compared for a binding consensus sequence. Two sites (bold) have a very conserved consensus inverted repeat sequence while the other two do not. The predicted ScbR binding consensus sequence is in bold italics. The ArpA binding consensus sequence and the BarA, FarA binding consensus sequence are also indicated. Note that a differently spaced inverted repeat related to the consensus is present in the *kasO*p site OB, and one element of this is shared with *scbA/R* site A.

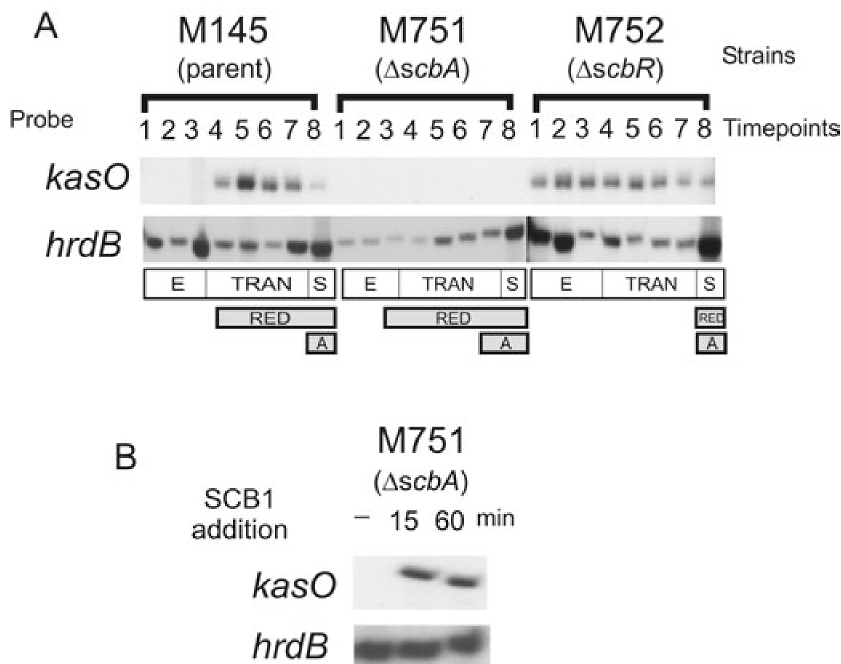

Fig. 5. Effects of mutations in *scbA* and *scbR* on *kasO* transcription.
A. S1 nuclease protection analysis of *kasO* and *hrdB* transcripts using RNA isolated from SMM liquid-grown cultures of *S. coelicolor* M145, M751 and M752. Numbers 1–8 denote the samples taken at OD_{450} (see *Experimental procedures*). The M145 and M752 RNA samples from time point 1 were the same as were used in Fig. 1, and for the microarray analysis (GC No. 2). E, TRAN and S indicate the exponential, transition and stationary phases of growth, respectively, and the shaded boxes labelled RED and A denote the production of undecylprodigiosin and actinorhodin respectively.
B. S1 nuclease protection analysis of *kasO* and *hrdB* transcripts using RNA isolated from Δ*scbA* at time zero (–) (OD_{450} 0.5), and 15 and 60 min after addition of SCB1. The same RNA sample for 60 min after addition of SCB1 was used for microarray analysis (addition No. 2).

resembles one of the previously identified ScbR targets, site R from the *scbA/R* promoter region (Fig. 4D; Takano *et al*., 2001). The consensus sequences found in the ArpA, BarA and FarA binding sites (Kinoshita *et al*., 1997; Onaka and Horinouchi, 1997) also show some similarity (Fig. 4D). Site OB of the *kasO* promoter also had palindromic sequences somewhat similar to the consensus, but with a 4 bp greater spacing.

kasO *is temporally expressed in M145, and is repressed by ScbR and induced by SCB1*

ScbR binds *in vitro* to the promoter regions of *kasO*, and SCB1 prevents this binding. To determine the effect of ScbR on *kasO* expression *in vivo*, S1 nuclease protection analysis was conducted on RNA from M145 (parent), M751 (Δ*scbA*) and M752 (Δ*scbR*). For time point 1 in M145 and M752, the same RNA samples that had been used in the microarray experiments (growth curve No. 2) were used. In M145, *kasO* transcription was undetectable during rapid growth, but was detected at transition phase, and declined as the cultures entered stationary phase. In contrast, in the *scbR* mutant, *kasO* expression was constitutive throughout growth, while *kasO* transcripts were not detected in the *scbA* mutant (Fig. 5A). These observations provided evidence that, *in vivo*, ScbR represses *kasO* and that SCB1 produced via *scbA* relieves this repression.

To test this hypothesis further, we also used S1 nuclease protection analysis to study the effect on *kasO* expression of adding chemically synthesized SCB1 at a final concentration of 31 ng ml^{-1} (128 μM) to a mid-exponential phase ($OD_{450} = 0.41$) culture of M751. RNA was isolated at 15 and 60 min after SCB1 addition. The RNA samples for no addition (time point 0) and 60 min after addition were the same samples used in the microarray experiments. There was a marked increase in the expression of *kasO* after the addition of SCB1, similar to the level detected in the parent (Fig. 5B). Thus, the relief of ScbR repression on *kasO* expression observed *in vivo* requires SCB1 produced via *scbA*.

Expression of the cryptic polyketide cluster is dependent on kasO

The role of *kasO* as a pathway-specific regulatory gene was determined by replacing most of the *kasO* coding sequence with an apramycin resistant cassette, yielding strain LW6. LW6 was constructed by the PCR targeting method (*Experimental procedures*) and confirmed by Southern hybridizations and by PCR (data not shown).

No obvious phenotype was detected with the *kasO* mutant and as the final product of the polyketide gene cluster is yet to be determined, expression of several genes in the cluster was assessed by RT-PCR. RNA was isolated from four different time points from LW6 grown in SMM liquid media. Four samples of RNA isolated from M145 for the expression studies of *kasO* was used for comparison. cDNA was synthesized from 2 μg of RNA (*Experimental procedures*) and cDNA equivalent to 0.1 μg

Table 3. List of primers used for RT-PCR experiments.

Primers	Primer sequence 5′–3′[a]	Fragment size[b] (bp)	Amplified region[c]
kasCrt1	gctcgacgaggcgtacgacaa	1040	SCO6273
kasCrt2	gcccgagccgcggcacgaaca		
kasSrt1	gtgctctctccgagaccttcg	741	SCO6276
kasSrt2	gacttgatgaagccgatgaag		
kasRrt1	cgtttctgctggaggtgctcg	540	SCO6279
kasRrt2	ccagctgttcctcgttcctgc		
scbRrt1	caggatgtgcttctgcagcag	492	SCO6265 (*scbR*)
scbRrt2	gcaggtcttcgagaagcaggg		
scbArt1	tctgcgtccgatgccaactcg	483	SCO6266 (*scbA*)
scbArt2	ggtagacttgaggactggtga		
hrdBrt1	gagtccgtctctgtcatggcg	547	SCO5820 (*hrdB*)
hrdBrt2	tcgtcctcgtcggacagcacg		

a. 5′ to 3′ primer sequences.
b. Size of PCR amplified product.
c. Description of the amplified promoter region. The SCO numbers are taken from the EMBL Accession No. AL645882. Gene names are also given. In all cases, template was total DNA of M145.

RNA was used in each PCR with different primers (Table 3). RNA was used as template for each primers as negative control and M145 total DNA was used as template for positive control.

While expression of the predicted biosynthetic genes, SCO6273, 6276 and 6279, in the cryptic polyketide cluster was detected in the parent at late transition phase, no or very little (SCO6276) expression was detected in LW6 (Fig. 6). A further 35 cycles of RT-PCR failed to reveal expression of SCO6273 and SCO6279 (data not shown). Expression of *scbR* and *scbA* was readily detected and effectively the same in both strains, as was expression of *hrdB* (encoding the major sigma factor for *S. coelicolor*). The expression profile of *scbA* corresponded well to the S1 nuclease experiments. However, expression of *scbR* was detected throughout growth using RT-PCR, while temporal expression was detected in the S1 nuclease experiments (Fig. 6). This may reflect the enhanced sensitivity of RT-PCR, and reflects the need for extensive controls when making quantitative measurements. Nevertheless, the results obtained with RT-PCR suggest strongly that *kasO* activates the expression of the cryptic polyketide gene cluster and that it is a pathway-specific regulatory gene. The negative controls showed no amplified product (data not shown) while the positive controls showed amplified products corresponding to the appropriate size for each primer (Fig. 6).

Replacement of *kasO* with the apramycin cassette may have had polar effect on the expression of the downstream gene, SCO6281. If SCO6281 is co-transcribed with *kasO*, then at this stage we cannot formally exclude the possibility that a reduction in the SCO6281 (which appears to encode a FAD-binding protein) expression might be responsible for the deficiency in SCO6273, 6276, 6279 transcript, although this seems unlikely.

Discussion

ScbR binds to the promoter region of a pathway-specific regulatory gene in a polyketide gene cluster

The use of newly available microarrays has led to the discovery that the cryptic polyketide gene cluster of *S.*

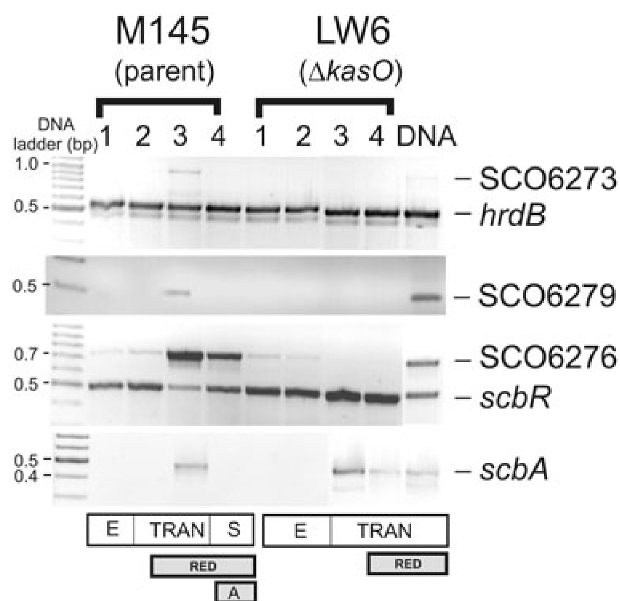

Fig. 6. Expression of several genes in the cryptic polyketide antibiotic cluster compared in the parent and the *kasO* mutant. RT-PCR using cDNA synthesized from RNA isolated from the parent (equivalent to the RNA samples used in Fig. 5A, No. 2, 4, 6 and 8), and from the LW6 mutant. Numbers 1–4 denote samples taken at various OD_{450} (see *Experimental procedures*). Amplified products are indicated on the right. The expected sizes of the amplified products are: SCO6273: 1.04 kb; *hrdB*: 0.55 kb; SCO6279: 0.54 kb; SCO6276: 0.74 kb; *scbR*: 0.49 kb; *scbA*: 0.48 kb. The template used for DNA positive control PCR was M145 total DNA for all primers. E, TRAN, and S indicate the exponential, transition and stationary phases of growth, respectively, and the shaded boxes labelled RED and A denote the production of undecylprodigiosin and actinorhodin respectively.

coelicolor is regulated by ScbR. We have identified a new target for ScbR by *in vitro* and *in vivo* analysis as the promoter region of *kasO*, shown to be a pathway-specific regulatory gene by the effects of a *kasO* deletion on expression of several genes of the pathway. In several γ-butyrolactone systems, there has been partial evidence to suggest that a γ-butyrolactone-binding protein regulates the expression of pathway-specific regulatory genes (Folcher *et al*., 2001; Stratigopoulos *et al*., 2002; Matsuno *et al*., 2004). However, this appears to be the first report to show that a γ-butyrolactone-binding protein directly represses a pathway-specific regulatory gene in an antibiotic biosynthesis gene cluster and that repression is relieved by the cognate γ-butyrolactone, leading to the expression of the pathway-specific regulatory gene and thus presumably to the expression of the antibiotic gene cluster.

ScbR binds to two regions in the promoter of *kasO*: site OA overlapping the –10 and –35 region of the transcriptional start site for *kasO*, and site OB at –222 nt to –244 nt. Site OA shows high similarity to the previously reported binding site R in the *scbA/R* promoter region sequence, which also functions to promote repression (Fig. 4D). On the other hand, site OB in the *kasO* promoter region shows only limited similarity to the consensus (ScbR bound to this site possibly binds to SCB1 less strongly than when ScbR is bound to site OA). However, there are no *in vitro* data at present to suggest that the binding of ScbR to site OB plays a role in activating the promoter, as may be the case for binding of ScbR to the second target (site A) in the *scbA* promoter (Takano *et al*., 2001).

There are reports where DNA binding sites were found *in vitro* and yet did not seem to have an effect on the expression of the genes *in vivo* (Kato *et al*., 2004). While this may eventually turn out to be true for site OB in the *kasO* promoter region, we did not observe any difference *in vitro* in the specificity of ScbR for the two binding sites by competition with unlabelled DNA fragments or by changing the amount of ScbR added (data not shown).

Further work will be needed to find out whether the product of the *scbR*-like gene (SCO6286; Table 3) in the polyketide cluster interacts with ScbR or binds to either of the ScbR binding sites in the *kasO* promoter, either independently of ScbR or in association with it.

The roles of ScbR and similar proteins in regulating secondary metabolism

The cryptic polyketide gene cluster contains at least three putative regulatory genes. Two, *kasO* and SCO6288, encode SARPs; while the other, SCO6286, is a homologue of *scbR* (K. Pawlik *et al*., in preparation). Interestingly, ScbR did not bind to the promoter regions of SCO6288, SCO6286 or any of the biosynthetic genes in the cluster: the sole target was the promoter region of *kasO*.

The role of each regulatory protein in the polyketide cluster is still to be determined by construction of appropriate mutants, and these experiments are hindered by the lack of knowledge of the compound whose production is directed by the cluster. The tylosin biosynthesis cluster (which also involves type I PKS genes) has a similar array of regulatory genes, notably two that encode SARP homologues showing high similarity to *kasO* and SCO6288, and two encoding γ-butyrolactone-binding protein homologues (Bate *et al*., 1999). Mutagenesis analysis by Bate *et al*. (2002) showed that *tylS* (encoding a SARP similar to that encoded by SCO6288) activates tylosin biosynthesis via *tylR*, while the role of the other SARP, encoded by *tylT* (a homologue of *kasO*), is not clear. *tylP* (a homologue of *scbR*) appears to be the main regulatory gene for tylosin biosynthesis and represses expression of both *tylQ* (a homologue of SCO6286; also encoding an ScbR-like protein) and *tylS* (Stratigopoulos and Cundliffe, 2002; Stratigopoulos *et al*., 2002). It is clear from our study that the details of the regulation of the polyketide gene cluster, although it involves similar elements, are different from those for the *tyl* cluster: if the second *scbR*-like gene has any regulatory role, it is likely to be as part of a regulatory cascade parallel to the one involving ScbR, rather than a sequential one, as in the tylosin biosynthesis.

Recently, the nucleotide sequences of a considerable number of gene clusters encoding the production of secondary metabolites have become available, including many in the genome sequences of *S. coelicolor* (Bentley *et al*., 2002) and *Streptomyces avermitilis* (Ikeda *et al*., 2003). At least 18 genes encoding homologues of γ-butyrolactone-binding proteins out of 36 are located in close proximity to antibiotic biosynthesis genes and have been shown to regulate antibiotic production (Table 4). It thus seems that γ-butyrolactones are strongly associated with the regulation of antibiotic production and that most of the γ-butyrolactone-binding proteins may well be pathway-specific regulatory proteins. Perhaps the *S. griseus* A-factor-binding protein, the first to be characterized in detail, is atypical in that it regulates multiple phenotypes, including morphological differentiation and the production of a yellow pigment, in addition to streptomycin production via AdpA; and ArpA does not appear to regulate pathway-specific regulatory genes directly (Kato *et al*., 2004). It is noteworthy that γ-butyrolactone receptor protein homologues have also been found in non-*Streptomyces* actinomycetes (Table 4), and that a recent report by Choi *et al*. (2003) identified γ-butyrolactones and receptor proteins from non-*Streptomyces* actinomycetes producing commercially important antibiotics. It will be

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interesting to determine whether those γ-butyrolactones are also involved in the regulation of antibiotic production.

Identification of further ScbR binding sites

SCB1 was identified by its ability to stimulate production of Act and Red, the pigmented antibiotics produced by *S. coelicolor* (Takano *et al.*, 2001). We have reported previously the possibility of ScbR regulating the production of Act and Red via a repressor for secondary metabolism. We have found one target of ScbR from the DNA microarray experiments described here. It is most probable that *kasO* is not the repressor for general secondary metabolism. However, the genes identified from the transcriptome analysis include seven that are unlikely to be involved in the synthesis of the polyketide that appears to be regulated by *kasO*. They are located far from this cluster and do not seem to be related to either potential precursor biosynthesis or transport of the compound. We have also shown in this report that ScbR cannot bind to the promoter region of these genes. Whether ScbR binds to the promoters of any other genes that in turn control the expression of these seven genes and also perhaps of Act and Red is yet to be determined. However, when DNA sequences homologous to the ScbR binding consensus sequence deduced from the two repressor sites were sought in the whole genome sequence of *S. coelicolor*, one such sequence (AACCGGNNNNNNGGTTTGT) was found in the promoter region of *orfB* (SCO6268), encoding a homologue of histidine protein kinases, one gene away from *scbA*. Further experiments are in progress to determine whether ScbR regulates *orfB*, and the effect, if any, of *orfB* on the expression of the seven unlinked genes identified by microarray analysis and on the expression of Act and Red biosynthesis genes.

This report explores one aspect of the microarray data, by using them as a preliminary guide to find the key target of ScbR in the polyketide gene cluster. Further microarray analysis using the *scbR* and *scbA* mutants at different time points of growth is in progress to analyse more sys-

Table 4. List of ScbR homologues from the EMBL data base.

Gene names/accession No.	Organism	Amino acid identity[a]	Reference
[b]**ScbR**/NP-630365	*S. coelicolor*	100%	Takano *et al.* (2001)
FarA/BAA21859	*S. lavendulae*	57%	Waki *et al.* (1997)
Orf82/NP-851504	*S. rochei* (plasmid pSLA2-L)	51%	Mochizuki *et al.* (2003)
[c]AlpZ/AAR30170	*S. ambofaciens*	48%	Pang *et al.* 2004
BarA/A57507	*S. virginiae*	47%	Kinoshita *et al.* (1997)
AvaR/NP-824882	*S. avermitilis* MA-4680	47%	
TarA/AAF06961	*S. tendae*	48%	Engel *et al.* (2001)
SabR/AAP8737	*S. ansochromogenes*	48%	Li *et al.* (2003)
SctR/BAC66444	*S. clavuligerus*	48%	Kim *et al.* (2004)
SpbR/AAK07686	*S. pristinaespiralis*	48%	Folcher *et al.* (2001)
TylP/T44586	*S. fradiae*	45%	Bate *et al.* (1999)
ArpA/BAA08617	*S. griseus*	41%	Onaka *et al.* (1995)
KsbA/BAD20229	*Kitasatospora setae*	38%	Choi *et al.* (2004)
AAM78022	*S. carzinostaticus* ssp. *neocarzinostaticus*	39%	
AAR90230	*Rhodococcus* sp. DK17 (plasmid PKD3)	34%	
SAV3702/NP-824879	*S. avermitilis* MA-4680	33%	
BarB/BAA23612	*S. virginiae*	37%	Kinoshita *et al.* (1997)
Orf79/NP-851501	*S. rochei* (plasmid pSLA2-L)	34%	Mochizuki *et al.* (2003)
MmfR/NP-639852	*S. coelicolor* (plasmid SCP1)	30%	
ApIW/AAR30167	*S. ambofaciens*	31%	Pang *et al.* (2004)
TylQ/T44588	*S. fradiae*	35%	Bate *et al.* (1999)
SCO6286/NP-630384	*S. coelicolor*	32%	
Orf74/NP-851496	*S. rochei* (plasmid pSLA2-L)	34%	Mochizuki *et al.* (2003)
CprB/NP-630180	*S. coelicolor*	32%	Onaka *et al.* (1998)
MAP0928/NP-959902	*Mycobacterium avium* ssp. *paratuberculosis* str. k10	32%	
SCO6323/NP-630417	*S. coelicolor*	29%	
CprA/NP-630409	*S. coelicolor*	32%	Onaka *et al.* (1998)
SAV2270/NP-823446	*S. avermitilis* MA-4680	31%	
ZP 00158065	*Anabaena variablilis* ATCC29413	30%	
JadR2/AAB36583	*S. venezuelae*	29%	Yang *et al.* (1995)
SAV2268/NP823444	*S. avermitilis* MA-4680	33%	
PBD2.026/NP-898641	*Rhodococcus erythropolis* (plasmid pBD2)	28%	
Alr4567/NP-488607	*Nostoc* sp. PCC 7120	30%	
AAR90230	*Rhodococcus* sp. DK17 (plasmid PKD3)	32%	
CprA MAP0969/NP-959903	*M. avium* ssp. *paratuberculosis* str. k10	28%	
BarZ/BAA96295	*S. virginiae*	34%	Kawauchi *et al.* (2000)

a. Amino acid identity with ScbR from BLAST searches performed at NCBI (http://www.ncbi.nlm.nih.gov).
b. Bold gene names denote those previously shown to cluster with antibiotic biosynthesis genes and regulate production of the cognate antibiotics.
c. Grey gene names represent those located in an antibiotic biosynthesis gene cluster.

tematically and to extend the genes that are controlled by the SCB signalling molecules.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Streptomyces coelicolor A3(2) strains M145 (Kieser *et al*., 2000), M751, M752 (Takano *et al*., 2001) and LW6 (this study) were manipulated as previously described (Kieser *et al*., 2000). *E. coli* K-12 strains JM101 (Sambrook *et al*., 1989) and ET12567 (MacNeil *et al*., 1992) were grown and transformed according to Sambrook *et al*. (1989). Vectors used were pIJ2925, pKC1132, pSET152 (Kieser *et al*., 2000), pBluescript SK$^+$ (Stratagene) and pDrive (Qiagen). pTE1 was constructed by cloning the *kasO* F+R (Fig. 4A; see Table 2 for primer sequences) PCR product into pDrive. SMM was as reported by Takano *et al*. (2001). SMMS is the agar (1.5%, w/v) version of the modified SMM. MS agar (Kieser *et al*., 2000) was used to grow cultures for preparing spore suspensions, and for plating out intergeneric conjugations of *Streptomyces* strains with *E. coli* ET12567 containing the RP4 derivative pUZ8002 (Flett *et al*., 1997).

RNA procedures, including microarray analysis and S1 nuclease protection analysis

RNA was isolated as described in Kieser *et al*. (2000) except that for microarray analysis samples were further purified by double chloroform extraction and eluted through an RNeasy column (Qiagen Cat. No. 75142). Samples for microarray analysis were harvested from SMM at OD_{450} 0.5 (14.0 h) for M145 and 0.46 (14.0 h) for M752 for growth curve No. 1 (GC1), and OD_{450} 0.38 (15.5 h) for M145 and 0.37 (15.5 h) for M752 for growth curve No. 2 (GC2). SCB1 was added to M751 at OD_{450} 0.41 (addition No. 1) and 0.41 (addition No. 2). *S. coelicolor* microarray (SC3, 4) design and production are described in Chater *et al*. (2002). SC3 arrays contained ≈6500 good (successful PCR) probes and the SC4 arrays 7333. In this article results from some SC4s are presented. Hybridization, wash conditions, scanning and image analysis were as described in Bucca *et al*. (2003) and http://www.surrey.ac.uk/SBMS/Fgenomics. Cy-3 labelled *scbR* mutant cDNA or parent M145 cDNA from time point 1 representing exponential growth was hybridized to the microarrays together with Cy-5 labelled M145 genomic DNA, or, in the case of SCB1 addition, time zero cDNA labelled with Cy-5 was compared with Cy-3 labelled cDNA from RNA isolated 60 min after SCB1 addition. Hybridizations were conducted in duplicate with each of the duplicate cDNA samples. After scanning with an Affymetrix 428 array scanner, the 16-bit TIFF images were processed with Imagene 4.2 software (BioDiscovery). Array data were analysed using GeneSpring (version 4.2, Silicon Genetics). Normalization was performed per spot: divide by control channel, per chip: normalize to 50th percentile. The statistical analysis tool in GeneSpring was used for further analysis and used a parametric test with global error model variances. This tool omitted any data with *P*-values of more than 0.05.

For each S1 nuclease reaction, 30 or 40 μg of RNA was hybridized in NaTCA buffer [Murray, 1986; solid NaTCA (Aldrich) dissolved to 3 M in 50 mM PIPES, 5 mM EDTA, pH 7.0] to about 0.002 pmol (approximately 10^4 Cerenkov counts min^{-1}) of the probes. To map the transcriptional start site of *kasO*, the oligonucleotide ksOR 5′-GTTGGC CTGCAACAGCAGGTA (which anneals within the *kasO* coding region) was uniquely labelled at the 5′-end with [^{32}P]-ATP using T4 polynucleotide kinase, and used in PCR with the unlabelled oligonucleotide ksOF 5′-GTGTATGT CACGGACGAGGAG (which anneals upstream of the *kasO* promoter) and cosmid SC1G7 DNA as template, to generate a 522 bp probe. For *hrdB* (Aigle *et al*., 2000) and *scbR/A* (Takano *et al*., 2001) the probes were made as previously described. Subsequent steps were as described by Strauch *et al*. (1991). RNA were isolated from liquid SMM-grown cultures of *S. coelicolor* M145 (parent), M751 (Δ*scbA*) and M752 (*scbR*) at OD_{450} of: (1) 0.36, 0.4, 0.39 (M145: 15.5 h, M751: 14.5 h, M752: 15.5 h), (2) 0.57, 0.53, 0.56 (17.0 h, 17.5 h, 17.4 h), (3) 0.78, 0.78, 0.79 (19.0 h, 19.0 h, 19.6 h), (4) 0.88, 0.84, 0.87 (20.5 h, 21.0 h, 21.4 h), (5) 1.04, 0.91, 1.0 (22.5 h, 22.0 h, 22.6 h), (6) 1.10, 0.92, 0.96 (23.5 h, 22.5 h, 24.4 h), (7) 1.12, 0.98, 1.14 (25.0 h, 23.0 h, 26.4 h), and (8) 1.10, 1.20, 1.28 (42 h, 42 h, 42 h).

Gel retardation assays and DNase I footprinting studies

For gel retardation experiments, oligonucleotides shown in Table 2 were used to generate PCR fragments for the corresponding gene promoter regions. Fragments were then labelled using a DIG Gel Shift Kit (Roche cat No. 1635352). Gel retardation, using ScbR-containing extracts from an *E. coli* overexpression construct, was carried out using the conditions described in Takano *et al*. (2001). In some cases, 1 μg of SCB1 or one of its stereoisomers was added before incubation and the mixture incubated for a further 10 min. Blotting and detection of the membrane was performed according to the manufacturer's conditions.

For DNase I footprinting studies, 50 pmol of oligonucleotide A1 and 4 (Fig. 4A; Table 1) were uniquely labelled on their 5′ ends with [^{32}P]-ATP using T4 polynucleotide kinase and used in PCR with unlabelled oligonucleotide A1 and 4, respectively, with cosmid SC1G7 (Redenbach *et al*., 1996) as a template, to generate a 434 bp DNA fragment. DNase I footprinting conditions were as described in Takano *et al*. (2001). In some cases, 1 μg of SCB1 was added to the reaction mixture before incubation and the mixture incubated for a further 10 min. Sequencing reactions were performed with the oligonucleotides used to amplify the PCR fragments as primers with pTE1 DNA as template, and using a dideoxy sequencing kit (Sequenase 7-deaza-dGTP DNA sequencing Kit, USB, product No. 70990).

Construction of a deletion mutant of kasO

A mutant *kasO* allele was constructed in which most of the *kasO* coding region (amino acids 17–530 out of 543) was deleted and replaced with an apramycin resistance gene using PCR targeting. Primers (5′-CGGATGCTCGGTCCAC

TCGAGGTGTTGTCCGGCGAGCAGATTCCGGGGATCCGT CGACC-3′) and (5′-TCAGATCGCCCCGCCTCCGGCGG GTGAGTCCTCGGCCGGTGTAGGCTGGAGCTGCTTC-3′) with 5′ ends overlapping the 5′ and 3′ ends of the *kasO* coding sequence, and 3′ (priming) ends were designed to amplify the apramycin resistance disruption cassette of pIJ773 (Gust *et al*., 2004). The mutant was constructed as described in Takano *et al*. (2003), except that the cosmid used to introduce the mutation into *Streptomyces* was SCAH10 (Redenbach *et al*., 1996). Deletions within *kasO* were confirmed by Southern hybridization (Kieser *et al*., 2000) using a PCR-generated probe (labelled with DIG DNA labelling kit, Roche) and also by PCR. Two independent colonies were isolated and one was designated LW6.

RT-PCR

RT-PCR was conducted using RNA isolated from LW6 grown in SMM liquid medium at four different time points: OD_{450} at: (1) 0.34 (17 h), (2) 0.54 (19 h), (3) 0.72 (21.5 h, produced Red) and (4) 0.90 (25 h) during the growth phase. RNA isolated for S1 nuclease protection analysis from M145 from time points (2) 0.57 (17.0 h) and (4) 0.88 (20.5 h), (6) 1.10 (23.5 h) and (8) 1.10 (42 h) was also used. cDNA was synthesized by following a protocol used for generating cDNA for microarray analysis. 2 μg of RNA was mixed with 0.34 μl of Random primers (Invitrogen) with dH_2O to a final volume of 15 μl, incubated at 70°C for 10 min and placed on ice. Six microlitres of 5× buffer (Invitrogen), 3 μl of DTT, 3 μl of dNTP mix (100 mM each), 2 μl of dH_2O were mixed with 1 μl of Superscript II (Invitrogen) and incubated at 25°C for 10 min, 37°C for 5 min, 42°C for 70 min to synthesize cDNA. One microlitre of the cDNA (equivalent to 0.1 μg of RNA) was used as template per PCR. PCR was conducted using Taq polymerase from Roche using the manufacturer's conditions (using Q buffer and dNTP with a final concentration of 200 μM). The programme was as follows: (1) 95°C, 5 min, (2) 95°C, 45 s, (3) 68°C, 45 s, (4) 72°C, 45 s, and (5) 72°C, 5 min repeating the two to four steps for either 20 cycles or 35 cycles. For negative control, RNA (1–2 μg) was used as template and for positive control, M145 total DNA (0.1 μg) was used as template.

Acknowledgements

We thank Helen Kieser for providing cosmids and Krzysztof Pawlik and the late Kataryzna Kuczek for personal communication of unpublished data. We also thank Andy Hesketh, David Hopwood and Krzysztof Pawlik for their comments on the manuscript. E.T. was supported from a grant from the Human Frontier Science Program RG0330/1998-M to M.B. at the John Innes Centre and by the Strukturford of the University of Tuebingen (III-H3-1415/547 01/02); H.K. and T.N. were supported by grants from the Ministry of Agriculture, Forestry and Fisheries of Japan; V.M., G.B., G.H. and C.P.S. were funded by grant FGT11407 from the Biotechnology and Biological Sciences Research Council (BBSRC); W.W. was funded by the BMBF (GenoMik); M.B. and K.F.C. were funded by a competitive strategic grant to the John Innes Centre from the BBSRC.

Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/mmi.mmi4543/mmi4543sm.htm
Appendix S1. Primary array data.

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