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Characterization of γ -Butyrolactone Autoregulatory Signaling Gene Homologs in the Angucyclinone Polyketide WS5995B Producer *Streptomyces acidiscabies*[∇]

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Organisms belonging to the genus *Streptomyces* produce numerous important secondary metabolites and undergo a sophisticated morphological differentiation program. In many instances these processes are under the control of γ -butyrolactone (GBL) autoregulatory systems. *Streptomyces acidiscabies* strain 84.104 produces the secondary metabolite aromatic angucyclinone polyketide WS5995B. In order to explore the role of GBL regulatory circuitry in WS5995B production and morphogenesis in *S. acidiscabies*, a gene cluster encoding GBL autoregulatory signaling homologs was identified and characterized. Two GBL receptor homologs, *sabR* and *sabS*, were found flanking a GBL synthase homolog *sabA*. Strains carrying mutations in *sabS* produced elevated levels of WS5995B and displayed conditional morphological defects reminiscent of defects seen in *Streptomyces bldA* mutants. Notably, *sabS* possesses a TTA codon predicted to be recognized by tRNA^{Leu}. *sabA* mutants produced higher levels of WS5995B than the wild-type strain but to a lesser extent than the levels of WS5995B seen in *sabS* mutants. Purified recombinant SabR and SabS were tested for their abilities to bind predicted AT-rich autoregulatory element (ARE) boxes within the *sabRAS* region. SabS did not bind any DNA sequences in this region, while SabR bound an ARE box in the region upstream of *sabS*. Quantitative reverse transcription-PCR analysis revealed higher levels of *sabS* transcript in *sabR* mutants than in the wild-type strain, suggesting that *sabS* expression is repressed by SabR. Based on these data, we propose that the *S. acidiscabies* *sabRAS* genes encode components of a signaling pathway which participates in the regulation of WS5995B production and morphogenesis.

Members of the genus *Streptomyces* are renowned for their morphological complexity as well as their capacity to produce a wide variety of important secondary metabolites, including polyketides and nonribosomal peptides. In many instances, the expression of structural genes encoding secondary metabolite pathway enzymes is regulated by the interplay of low-molecular-weight hydrophobic, membrane-diffusible γ -butyrolactone (GBL) autoregulator compounds with specific cognate GBL receptors. GBL autoregulator synthesis requires the autoregulator GBL synthase, and as GBL is produced, intracellular GBL levels increase. GBL receptors are transcriptional regulators belonging to the TetR superfamily of transcription factors (34, 37). These proteins possess helix-turn-helix (HTH) DNA binding domains which recognize AT-rich autoregulatory element (ARE) DNA sequence targets (34).

Typical GBL signaling relies on changes in ARE-receptor interactions in response to increasing intracellular levels of GBL. The best-studied GBL signaling system is that of *Streptomyces griseus* (35). The GBL receptor ArpA binds target DNA sequence in the upstream region of *adpA*, resulting in repression of *adpA* expression by obstructing RNA polymerase access to *adpA* promoter elements. Biosynthesis of the *S. griseus* GBL A factor (2-isocapryloyl-3-*R*-hydroxymethyl- γ -butyro-

lactone) requires the product of the autoregulator gene *afsA*. As A factor reaches sufficient levels within the cell, the binary ArpA/A-factor complex dissociates from the target nucleotide sequence, thereby allowing *adpA* expression. AdpA is required for expression of a set of genes which constitute the AdpA regulon. This includes genes required for morphological differentiation, streptomycin biosynthesis, grixazone production, and the production of other metabolites (16).

Other examples of GBL signaling systems in streptomycetes which have been described include the SCB1 system of *Streptomyces coelicolor*, which regulates production of both actinorhodin and undecylprodigiosin (42); the control of blue pigment and nucleoside antibiotic biosynthesis by the IM-2-C6 system of *Streptomyces lavendulae* (23); and the virginiae butanolide control of virginiamycin production by *Streptomyces virginiae* (31). Additionally, in at least some cases, secreted compounds other than those produced by cognate GBL synthases are capable of activating GBL signaling pathways in *Streptomyces*. For example, Birkó et al. (3) have reported that a 324-amino-acid peptide known as factor C produced by certain streptomycetes is capable of restoring phenotypic defects of A-factor-deficient mutants of *S. griseus*.

Streptomyces acidiscabies strain 84.104 represents an interesting bacterium to explore GBL signaling phenomena since it produces both nonribosomal peptide and polyketide secondary metabolite compounds. These include the nonribosomal cyclic dipeptide thaxtomin A (25) and the angucyclinone polyketide WS5995B (17, 21). WS5995B (Fig. 1) exhibits antimicrobial

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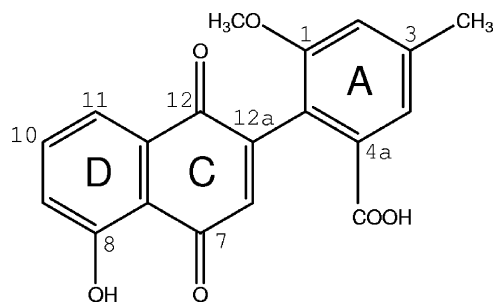


FIG. 1. Structure of WS5995B.

activity (17; also our unpublished results), whereas thaxtomin A displays phytotoxic activity against susceptible plant hosts (13, 29). Other phytopathogenic streptomycetes are known in addition to *S. acidiscabies* which produce thaxtomin toxins. Among these, it was recently shown that *Streptomyces scabies* strain 87.22 possesses an AraC/XylS-type transcriptional regulator to control expression of thaxtomin synthetase structural gene expression in response to cellobiose (18). The regulation of thaxtomin biosynthesis has not been explored in *S. acidiscabies*, and, interestingly, *S. acidiscabies* is the only currently available organism known to produce WS5995B.

The widespread utilization of GBL signaling systems in the regulation of morphogenesis and secondary metabolism among streptomycetes along with the poorly understood regulatory mechanisms governing production of the nonribosomal peptide thaxtomin and the type II polyketide WS5995B in *S. acidiscabies* prompted us to explore and characterize the possible roles played by GBL signaling in the production of these two distinct types of secondary metabolites in *S. acidiscabies*.

Our results reported here describe the identification and characterization of a GBL signaling system consisting of the GBL synthase homolog *sabA* and two adjacent flanking genes, *sabR* and *sabS*, encoding GBL receptors. The results of genetic and biochemical studies described here reveal a role for this GBL system in regulating production of WS5995B. In our analysis of GBL mutants and metabolite production profiles, we find no changes in thaxtomin production relative to the wild-type strain, suggesting that the GBL system described here does not play a role in the biosynthesis of thaxtomin or its regulation. In addition to GBL's effect on WS5995B production, we also report a conditional morphological defect in strains carrying mutations in the GBL receptor *sabS*, suggesting that SabS plays a role in morphogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth, and culture conditions. Bacterial strains and plasmids used in this study are given in Table 1. *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium. Antibiotics ampicillin, kanamycin, apramycin, and chloramphenicol, when used for selection in *E. coli*, were added to medium at final concentrations of 100, 50, 100, and 30 µg/ml, respectively. Growth and transformation procedures for *E. coli* strains were carried out using standard methods (40). *S. acidiscabies* cultures were routinely grown at 30°C in liquid tryptic soy broth medium in 250-ml baffled flasks containing 20 to 30 ml of medium or on ISP2 solid medium (41). For analysis of WS5995B production, *Streptomyces* cultures were grown at 30°C in liquid SGM (*Streptomyces* growth medium) or on solid SGM (21) without added calcium carbonate. Growth of *Streptomyces* strains in SGM was measured by determining wet weights of cells that had been collected by vacuum filtration. Following conjugal transfer of mutant alleles or complementing plasmids from *E. coli* donors, *Streptomyces* transconjugants were selected on modified ISP4 medium. Modified ISP4 medium was prepared as previously described (7, 41), except that calcium carbonate was omitted, and the medium was amended with 0.5 g/liter yeast extract and 1 g/liter tryptone. Additionally, after an autoclaving step, 0.1 volume of sterile 400 mM MgCl₂ was added to medium to give a final MgCl₂ concentration of 40 mM

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties and/or function	Reference or source
Strains		
<i>S. acidiscabies</i> 84.104	Wild-type WS5995B producer	21
SabR4–9	<i>S. acidiscabies</i> 84.104 Δ <i>sabR</i> GBL receptor mutant	This study
SabA5	<i>S. acidiscabies</i> 84.104 Δ <i>sabA</i> GBL synthase mutant	This study
AKP938	<i>S. acidiscabies</i> 84.104 Δ <i>sabS</i> GBL receptor mutant	This study
<i>E. coli</i> DH5 α MCR	Cloning host; F [–] <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 Δ <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) <i>U196 endA1 recA1 deoR thi-1 phoA supE44</i> λ [–] <i>gyrA96 relA1</i>	13
<i>E. coli</i> BW25113	Strain used for PCR targeted mutagenesis; Δ <i>araBAD hsdR514</i>	8
<i>E. coli</i> S17-1	Donor strain for conjugal transfer; <i>thi pro hsdR hsdM⁺ recA RP4 tra</i>	13
<i>E. coli</i> BL21 λ DE3	Strain for recombinant protein expression	Novagen
Plasmids		
pUC19	General cloning vector	13
pGBLBP1	pUC19 carrying <i>sabRAS</i> genes on a 7-kb KpnI fragment	This study
pOJ260	Suicide vector for integration in <i>Streptomyces</i> ; <i>aac(3)IV</i> oriColE1 RP4 <i>oriT lacZα</i>	13
pALE21	pOJ260 carrying <i>sabRAS</i> genes on 7-kb KpnI fragment	This study
pKD46	Vector carrying arabinose-inducible λ Red recombinase; <i>bla araC</i> γ β <i>exo repA101ts</i> oriR101	8
pKD3	Template plasmid carrying Flp recognition target-flanked <i>cat</i> gene for creating PCR mutagenesis cassette	8
pCP20	<i>bla cat</i> thermal induction of FLP synthesis	8
pIJ86	<i>Streptomyces</i> complementation plasmid; oriColE1 SCP2* <i>aac(3)IV ermE*</i> p ^a	Mervyn Bibb
pIJ86: <i>sabS</i>	pIJ86 carrying wild-type <i>sabS</i> allele under transcriptional control of <i>ermE*</i> p	This study
pET26b	T7 RNA polymerase-dependent recombinant protein expression vector	Novagen
pET26b:: <i>sabR</i>	pET26b carrying <i>sabR</i> encoding GBL receptor SabR	This study
pET26b:: <i>sabS</i>	pET26b carrying <i>sabS</i> encoding GBL receptor SabS	This study

^a *ermE**p, *ermE** promoter.

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence (5'–3')
GBL-F	CAAGCAGGAGCGCGSRTCCGSAC
GBL-R	CCTTGGACTGCAAGTGGAAAGTASA
sabRex-F	AAGGATCATATGGCTAAACAGGAGCGC
sabRex-R	AAGGATCTCGAGCGAAGCCACCTTTCGGG
sabSex-F	TTCCTTCATATGGCGAGGCAGTTACGC
sabSex-R	TTCCTTCTCGAGGGACACGCACGCCGCCGT
ARE-F	AGTCGGCAGTCTTCGAGTGT
ARE-R	GCTCGTCGATTCGTAACCAT
AREdupF	TGAGAAACAACGGCATGACCCGTTCTTTT ACCG
AREdupR	CGTAAAGAACGGGTCATGCCGTTTGT CTCA
sabRdelF	CCAAGCCATCGTGAAGCGGCCGGTGAGG TTTTCGACGACCGTTACATGTGTAGG CTGGAGCTGCTC
sabRdelR	CGAACGGCACCTCGCCAGCACCGCCT GCGCCAGCGCTCCTTGACCATATGAAT ATCCTCCTTAG
sabAdelF	GTTCTCCACCGCGCAGCCCTCGCCGAAAC GTTTCTACCGGTGGGAGCTGTGTAGGC TGGAGCTGCTC
sabAdelR	CGGCACCGGCCGACCTCCAACCGGTGCGG ATCCGTGGTACTCGAAGGCATATGAAT ATCCTCCTTAG
sabSdelF	ATGGCGAGGACGTTACGCGCCGAACAGAC CCGCGCGACGATCATACGGCGTGTGTA GGCTGGAGCTGCTC
sabSdelR	TCAGGACACGCACGCCCGTGTATCTCCCG CTCCAGCTCGACCCAGGCTCAGCATAT GAATATCCTCCTTAG
sabRcomplF	ACCTAAGCTTATCGGCCAACTCGGTCC
sabRcomplR	AACCAAGTACTACGTTTTCTTCTCCGC
sabAcomplF	ACCTAAGCTTTGTTTCGGCCCGATCA
sabAcomplR	AACCAGATCTCGGGTTCATGCCGTTTGT
sabScomplF	ACCTAAGCTTTGTGTACAGGGGGTGA
sabScomplR	AACCAAGTCTCGATCCGAACAGCGCCG
hrdBF	GAAGACGCTCCGAARAARACNRC
hrdBR	GGGTGGCGCAGCTTGSWCATNGTYTT
hrdBFrt	CTGGATGAGGTCCAGGAAGA
hrdBRrt	CCGTTCAAGGACTACCTCAA
sabRFrt	GTACTTCACTTCCCGTCCA
sabRRrt	CAACAAGGAACTGGTCAGCA
sabAFrt	ACACCCCGTTTTCTTCGAC
sabARrt	GACCCACACTCGAAGACTG
sabSFrt	ATCTGTTCCAGCCATGTT
sabSRrt	CGCTTCGAGGGAGGAGTAG
sabSQRTF	GCGCCTGTACTTCCACTT
sabSQRTR	TGGGACTGGAGTTCATGAT
hrdBQRTF	CTCTTCTGGACCTATCCA
hrdBQRTR	GGCGTACGTGGAGAATTGT

in agar medium. Apramycin sulfate and nalidixic acid were each added to ISP4 medium at 25 µg/ml to select for *Streptomyces* transconjugants and to counter-select *E. coli* donors, respectively.

Cloning of GBL signaling genes *sabRAS* in *S. acidiscabies* 84.104. All oligonucleotide primers used in this study are presented in Table 2. Consensus degenerate hybrid oligonucleotide primers (39) were selected using protein alignments of previously characterized GBL receptors. The oligonucleotide primer pair GBL-F/GBL-R (Table 2) was used to amplify GBL receptor gene fragments from *S. acidiscabies* 84.104 genomic DNA using "touchdown PCR" as previously described (13). Cloned amplification products were sequenced and used as probes for the identification of complete genes and flanking regions from gene libraries using colony hybridization methods (13). The nucleotide sequence of the *sabRAS* region was determined on both strands of a 7-kb KpnI insert in plasmid vector pUC19 (pGBLBP1) using a primer walking approach. Deduced GBL receptor and synthase protein sequences were aligned using MUSCLE, version 3.7. Alignments were refined using Gblocks, version 0.91b, and phylogenies were constructed with the maximum-likelihood method using PhyML,

version 3.0. Phylograms were visualized and edited using TreeDyn, version 198.3 (9).

Mutagenesis and complementation of *sabRAS* genes. The 7-kb KpnI fragment from pGBLBP1 was cloned into the KpnI site of plasmid vector pOJ260, and the resulting plasmid (pALE21) was introduced into *E. coli* strain BW25113 carrying plasmid pKD46, encoding bacteriophage lambda Red recombinase. Cultures carrying both of these plasmids were grown in LB medium containing apramycin and ampicillin. BW25113(pALE21/pKD46) was grown in SOB medium containing 1 mM L-arabinose to induce expression of Red recombinase, as previously described (8). Oligonucleotide primer pairs sabRdelF/sabRdelR, sabAdelF/sabAdelR, and sabSdelF/sabSdelR (Table 2) were selected to generate in-frame deletions of *sabR*, *sabA*, and *sabS* on plasmid pALE21, respectively. These primers were used to amplify the chloramphenicol acetyltransferase gene from template plasmid pKD3, with resulting products carrying 5' ends with homology to *sabR*, *sabA*, or *sabS* in pALE21 (8, 11). Gel-purified PCR products were electroporated into washed suspensions of BW25113(pALE21/pKD46) as described previously (8), and transformed cells carrying mutagenized pALE21 were selected on LB agar containing chloramphenicol. Overnight cultures of resulting chloramphenicol-resistant colonies were grown in LB medium containing chloramphenicol, and plasmids were extracted from these cultures. Plasmids thus obtained were used to transform suspensions of competent *E. coli* DH5α cells to chloramphenicol resistance. Plasmids extracted from these transformants were used to transform BW25113(pCP20) to apramycin resistance in order to excise the chloramphenicol resistance gene as described previously (8).

pALE21 derivatives carrying deletions in *sabR*, *sabA*, and *sabS* were used to transform either *E. coli* strain S17-1 or the ET12567(pUZ8002) strain to apramycin resistance. Resulting transformants were used for intergeneric conjugal matings with *S. acidiscabies* 84.104 recipient strains as previously described (13), with the exception that mating mixtures were plated on 25 ml of modified ISP4 medium instead of AS-1 medium. Following overnight growth of mating mixtures at 30°C, transconjugants were selected by overlaying plates with 5 ml of soft nutrient agar containing nalidixic acid and apramycin sulfate (150 µg/ml each) to give final concentrations of 25 µg/ml in agar medium. Incubation of plates was continued at 30°C for 3 to 5 days until transconjugants were visible on agar surface. Transconjugant colonies were then transferred to ISP2 medium containing nalidixic acid and apramycin. Liquid cultures of transconjugants were grown nonselectively in tryptic soy broth for two or three serial transfers to allow for loss of integrated plasmid. PCR assays were used to screen apramycin-sensitive derivatives for loss of plasmid and retention of mutant alleles. These mutants were used to study the roles of GBL signaling genes in secondary metabolite biosynthesis and morphological differentiation on solid medium.

In order to verify linkage of mutant phenotypes with deleted GBL alleles, the primer pairs sabRcomplF/sabRcomplR, sabAcomplF/sabAcomplR, and sabScomplF/sabScomplR were used to amplify the wild-type *sabR*, *sabA*, and *sabS* open reading frames (ORFs), respectively, from *S. acidiscabies* 84.104 genomic DNA using high-fidelity *Pfu* polymerase (New England Biolabs). Amplification products were purified and digested with restriction enzymes HindIII and BglII. Plasmid pIJ86 was also digested with HindIII and BglII, and the ORFs encoding these GBL signaling genes were ligated into pIJ86 using T4 DNA ligase (New England Biolabs). The resulting plasmids, pIJ86::*sabR*, pIJ86::*sabA*, and pIJ86::*sabS*, harboring wild-type genes under the transcriptional control of the *ermE** promoter were used to transform *E. coli* S17-1 or ET12567(pUZ8002), and conjugations were performed using these strains as donor and the Δ *sabR*, Δ *sabA*, or Δ *sabS* strain as recipient. Resulting transconjugants were selected on ISP4 medium amended with apramycin and nalidixic acid.

Analysis of WS5995B production. *S. acidiscabies* 84.104 wild type and GBL deletion mutants were grown on ISP2 medium, and mycelial fragments were used to inoculate tryptic soy broth liquid medium (20 ml in 250-ml baffled Erlenmeyer flasks). Cultures were grown at 30°C and 180 rpm for approximately 20 h. Two milliliters of these cultures was used to inoculate 280 ml of SGM in 2.8-liter baffled Fernbach flasks. Culture medium was collected at various times, and cells were removed by vacuum filtration. Wet cell weight determinations of cultures were made, and filtrates were extracted using Strata-X solid-phase cartridges as described by the manufacturer (Phenomenex).

Extracted metabolites were dissolved in methanol and investigated by thin-layer chromatography (TLC) analysis on silica gel plates (250 µm; Whatman). UV and visible absorbance spectra were collected using a Beckman DU640B spectrophotometer. Quantitative high-performance liquid chromatography analysis was done using a Hewlett Packard 1090 liquid chromatograph equipped with diode-array detector and a Phenomenex C₁₈ Luna (5 µm; 150 by 4.6 mm) column. Detection wavelength range was from 200 to 600 nm. Solvent A was H₂O · H₃PO₄ at 99.9:0.1; solvent B was 100% CH₃CN. Samples of extracts were injected and chromatographed at a 1 ml/min flow rate. Extract components were

eluted with a linear gradient of 25% B to 60% B over 21 min. Identification and quantification of WS595B in culture filtrate extracts were accomplished using authentic WS595B standard preparations (21).

Cloning, expression, and purification of recombinant SabR and SabS. The *sabR* and *sabS* ORFs were amplified from *S. acidiscabies* 84.104 genomic DNA using high-fidelity *Pfu* polymerase and the oligonucleotide primer pairs sabRex-F/sabRex-R (for SabR expression) and sabSex-F/sabSex-R (for SabS expression) (Table 2). Amplification reaction products were purified and digested with restriction enzymes NdeI and XhoI. The expression vector pET26b (Novagen) was also digested with NdeI and XhoI, and *sabR* and *sabS* ORF restriction digestion products were ligated into the vector to produce recombinant protein expression constructs pET26b::*sabR* and pET26b::*sabS*. Competent cells of *E. coli* strain BL21(ΔDE3) were transformed with these plasmids, and transformants were selected on LB agar containing kanamycin. Transformants were grown in 500 ml of LB medium containing kanamycin at 30°C. When the culture reached an optical density at 600 nm of approximately 0.6, protein expression was induced with the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 80 μM. Cultures were grown for an additional 6 h and then harvested by centrifugation. Cell pellets were washed once in buffer containing 20 mM sodium phosphate, 500 mM NaCl, and 20 mM imidazole, pH 7.4, and then resuspended in 1 to 2 ml of the same buffer containing 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride. Cell suspensions were disrupted by passage through a French pressure minicell at 16,000 lb/in². The cell lysate was centrifuged at 20,000 × *g* for 30 min, and the resulting soluble protein fraction was loaded onto a 1-ml HisGraviTrap column (GE Healthcare). Protein fractions were eluted with 20 mM sodium phosphate–500 mM NaCl, pH 7.4, containing increasing amounts of imidazole. Fractions were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE), and the fractions containing pure SabR or SabS (as judged by electrophoretic homogeneity) were pooled and desalted on PD-10 desalting columns (GE Healthcare). Proteins obtained in this manner were stored at –20°C or –70°C in 50 mM Tris-Cl, pH 7.6, 200 mM NaCl, 1 mM DTT, and 50% glycerol. Protein determinations were made using a Bradford assay with bovine serum albumin as the standard.

Electrophoretic mobility shift assays. DNA fragments encompassing ARE sequences within the *sabRAS* region were amplified from template plasmid pGBLBP1. The oligonucleotide primer pair ARE-F/ARE-R was used to amplify a 208-bp DNA fragment encompassing the upstream region of *sabS*. ARE-F (40 pmol) was first end labeled in a separate reaction using [γ -³²P]ATP (6,000 Ci/mmol; PerkinElmer) and polynucleotide kinase (Roche) according to the manufacturer's recommendations. Labeled primer products were separated from unincorporated nucleotide using Micro Bio-Spin columns (Bio-Rad) and used in PCR along with oligonucleotide primer ARE-R, plasmid template pGBLBP1, and high-fidelity *Pfu* polymerase. Amplified labeled PCR product was separated from excess primer using QiaQuick PCR spin columns (Qiagen). DNA binding assays contained 2.5 × 10³ cpm of labeled DNA, 50 ng of calf thymus DNA, 50 mM NaCl, 10 mM MgCl₂, 10 mM Tris-Cl, 1 mM DTT, and 50 μg/ml bovine serum albumin, pH 7.4. Various amounts of recombinant SabR or SabS were added to the reaction tubes. For competition experiments, unlabeled competitor duplex DNA corresponding to ARE sequence was prepared by briefly heating and annealing stoichiometric amounts of complementary oligonucleotides AREdupF/AREdupR and adding these to DNA binding reaction mixtures. Binding reactions (10 μl) were carried out for 20 min at 30°C, after which reaction products were electrophoresed through nondenaturing 5% polyacrylamide gels in 0.5× Tris-borate-EDTA buffer (40). Gels were dried under vacuum and exposed to a phosphorimager screen (Amersham Biosciences). Exposed screens were scanned using a Typhoon Trio+ Variable Mode Imager (Amersham Biosciences); band intensities of scanned screens were quantified using ImageQuant TL software (Amersham Biosciences). Experiments were repeated at least four times for each protein and ARE substrate combination.

RNA extraction and reverse transcriptase PCR assays. Cultures of *S. acidiscabies* wild-type 84.104 and of *sabR*, *sabA*, and *sabS* mutants were grown in tryptic soy broth or SGM to mid-log phase, and cells were collected by centrifugation. Cell pellets were briefly homogenized using a tissue grinder and treated with lysozyme. Total RNA was extracted from cells using an RNeasy minikit following the manufacturer's guidelines (Qiagen). Following extraction, RNA samples were treated with RNase-free DNase I (New England Biolabs). Reverse transcription reactions were carried out using Superscript II reverse transcriptase (Invitrogen) and reverse transcriptase primers sabRRrt, sabARrt, and sabSRrt (Table 2). PCR amplification of DNase-treated samples prior to cDNA synthesis yielded no amplification products, indicating that nucleic acid PCR amplification products observed following reverse transcription were produced solely from cDNA templates derived from RNA. Following cDNA synthesis, reverse transcription reaction products were used for PCR amplification using the primer

pairs sabRrt/sabRRrt, sabARrt/sabARrt, and sabSRrt/sabSRrt. PCR was carried out for 30 cycles, and amplification products were electrophoresed through 1% agarose gels in Tris-acetate-EDTA buffer. PCR amplification product band intensities on gels were imaged using a VersaDoc system (Bio-Rad) and quantified using Quantity One software. Normalization of amplification products from cDNA templates using GBL primers was done using the *hrdB* gene encoding the principal sigma factor of *S. acidiscabies* 84.104. *hrdB* was cloned from *S. acidiscabies* 84.104 genomic DNA using degenerate oligonucleotide primers designed from alignments of conserved regions of principal sigma factors from *S. coelicolor* (SC05820), *Streptomyces avermitilis* (SAV_2444), and *S. griseus* (EMBL accession X75952) (Table 2). The deduced amino acid sequence of the cloned *S. acidiscabies* 84.104 *hrdB* gene displayed 95% identity with *S. coelicolor* HrdB (not shown). The oligonucleotide primer pair hrdBFRt/hrdBRRt was used for amplification of *hrdB* cDNA from RNA samples.

Quantitative reverse transcription-PCR (qRT-PCR) was performed using an Applied Biosystems 7500 real-time PCR system and SYBR green Quantitative RT-PCR Kit with Moloney murine leukemia virus reverse transcriptase, RNase inhibitor, and JumpStart *Taq* DNA polymerase (Sigma). Specific primers used for amplification of *sabS* (sabSQRT) and *hrdB* (hrdBQRT) are given in Table 2. Amplicon specificity was checked using qRT-PCR dissociation curve analysis. The relative increase in *sabS* expression in wild-type and *sabR* mutant strains was determined using the Relative Quantification Method (Applied Biosystems 7500 System). Briefly, threshold cycle (*C_T*) values were normalized to *hrdB* mRNA levels for RNA samples from each strain, and mean relative expression ratios were calculated using the ΔΔ*C_T* method. Values given for the relative increase in expression represent the means of three independent experiments.

Nucleotide sequence accession number. The nucleotide sequence of the *sabRAS* region described here has been deposited in the NCBI database under accession number FJ821515.

RESULTS AND DISCUSSION

Identification and characterization of GBL signaling genes in *S. acidiscabies* 84.104. Degenerate PCR primers were designed to amplify 5' ends of genes encoding GBL receptor proteins from the WS595B and thaxtomin producer *S. acidiscabies* strain 84.104. GBL receptor genes encode an N-terminal region unique to GBL receptors and the HTH DNA binding domain representative of members of the TetR transcriptional regulator superfamily (14, 37). Amplification using oligonucleotide primers selected to anneal to these DNA sequences resulted in the production of an expected ~150-bp fragment from *S. acidiscabies* 84.104 genomic DNA (data not shown). The amplification product was cloned and sequenced; the deduced translation product displayed a high degree of similarity to N-terminal sequences of GBL receptors carrying N-terminal HTH domains. The amplification product was radiolabeled and used to obtain the complete sequence as well as flanking sequences from an *S. acidiscabies* genomic library. A library clone containing a 7-kb KpnI fragment and carrying GBL receptor and autoregulator homologs was identified and sequenced (Fig. 2).

Sequence analysis identified the GBL receptor gene homolog *sabR*. SabR has a predicted anhydrous molecular mass of 23.47 kDa. The predicted SabR translation product is 48% identical to the *S. virginiae* butanolide receptor BarA (22). The translation start of *sabR* is separated from the translation start of the divergently transcribed GBL synthase autoregulator homolog *sabA* by 89 nucleotides (Fig. 2). The SabA gene product has a predicted anhydrous molecular mass of 36.64 kDa. SabA is 48% identical to the BarX autoregulator of *S. virginiae* (20). The 3' end of the *sabA* coding region is separated from the 5' end of a second GBL receptor gene homolog, *sabS*, by 33 nucleotides (Fig. 2). Two possible translation initiation codons were identified for SabS. Translation from the 5'-most initia-

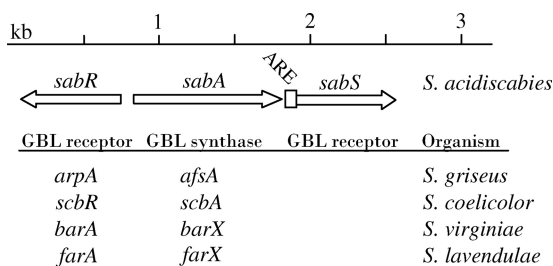


FIG. 2. Genetic organization of the *sabRAS* region in *S. acidiscabies* 84.104. GBL receptor gene homologs *sabR* and *sabS* and GBL synthase homolog *sabA* are indicated by arrows below scale, given in kilobases. ARE on the diagram represents a 26-bp ARE DNA sequence (not drawn to scale) bound by SabR (Fig. 7). For comparative purposes, a partial list of characterized receptor/synthase partners from other streptomycetes is given below the *sabRAS* gene map although these gene pairs are not necessarily organized in the same manner as shown in figure.

tion codon would result in a protein with a predicted anhydrous molecular mass of 25.44 kDa and is 70% identical to CprB of *S. coelicolor* (32). A second in-frame translation initiation codon is also observed (see Fig. 6), and translation from this codon would result in the production of a protein with anhydrous molecular mass of 24.12 kDa. Both initiation codons are preceded by appropriately spaced Shine-Dalgarno sequences complementary to the 3' end of *S. acidiscabies* 16S rRNA (45).

Both GBL receptor homologs SabR and SabS have distinct N-terminal HTH motifs as well as conserved residues thought to constitute a hydrophobic GBL binding pocket, based on structural studies of the GBL receptor CprB (32). Protein sequence alignments using SabA and other GBL synthases required for GBL biosynthesis, most notably AfsA, showed

that SabA contains conserved residues involved in GBL biosynthesis (19).

In order to evaluate the evolutionary relationships of SabR, SabS, and SabA to other GBL signaling gene components, phylogenetic trees were constructed using these proteins and homologous proteins, and these results are presented in Fig. 3. Inspection of the tree in Fig. 3A shows SabR to be somewhat related to the GBL receptors TylP and TylQ while SabR is distantly related to SabS. SabS, on the other hand, is most closely related to CprA. Results presented in Fig. 3B reveal SabA to be closely related to a possible GBL synthase, AAM78023.1, found in *Streptomyces carzinostaticus* (W. Liu et al., unpublished results). The phylogenetic data presented here largely support results of previous phylogenetic analyses suggesting that GBL receptor genes and their cognate synthases are evolving independently with respect to one another (34).

Characterization of *sabR*, *sabA*, and *sabS* mutants. In order to study the roles of *sabRAS* genes in *S. acidiscabies*, in-frame deletion mutants of each gene were constructed using a modification of the bacteriophage lambda Red recombinase method developed by Datsenko and Wanner (8) and later adapted to *Streptomyces* (11). Our approach involved PCR amplification of the template plasmid pKD3 *cat* antibiotic resistance gene and electroporation mutagenesis of a derivative of the suicide plasmid vector pOJ260 (pALE21) which carries the cloned mutagenesis target genes and the origin of conjugal DNA transfer *oriT* (2). Previous conjugation experiments using *S. acidiscabies* utilized AS-1 medium for plating of conjugation mixtures (12, 13). More recent conjugation studies with *S. acidiscabies* in our lab have shown that improved results could be obtained using ISP4 medium amended with yeast extract and 40 mM MgCl₂. Experiments using this medium resulted in 10- to 30-fold increases in observed transconjugant frequency.

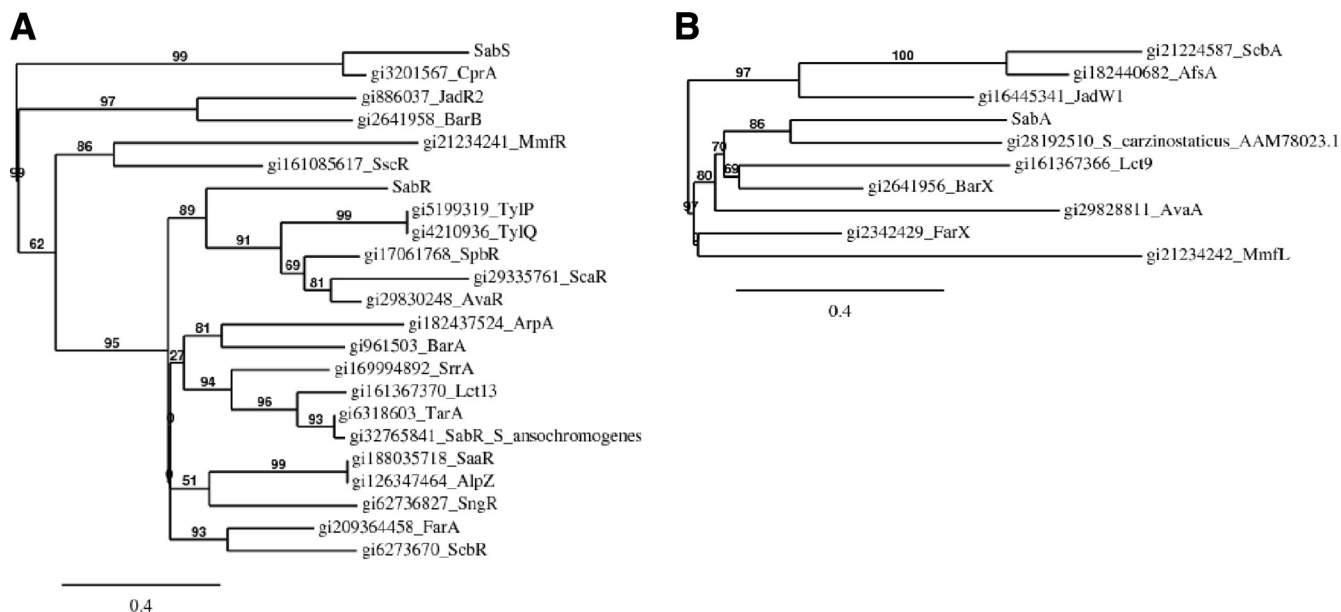


FIG. 3. Phylogenetic analysis of *sabRAS* gene products. (A) Phylogram showing relationships between *Streptomyces* GBL synthases and SabA. (B) Phylogram showing relationships between *Streptomyces* GBL receptors and *S. acidiscabies* SabS and SabR proteins. Proteins are represented by gene identification (gi) numbers and names in the NCBI/GenBank protein sequence database; SabR_S.ansochromogenes represents GBL receptor SabR found in *Streptomyces ansochromogenes*. Bootstrap values are indicated at branches.

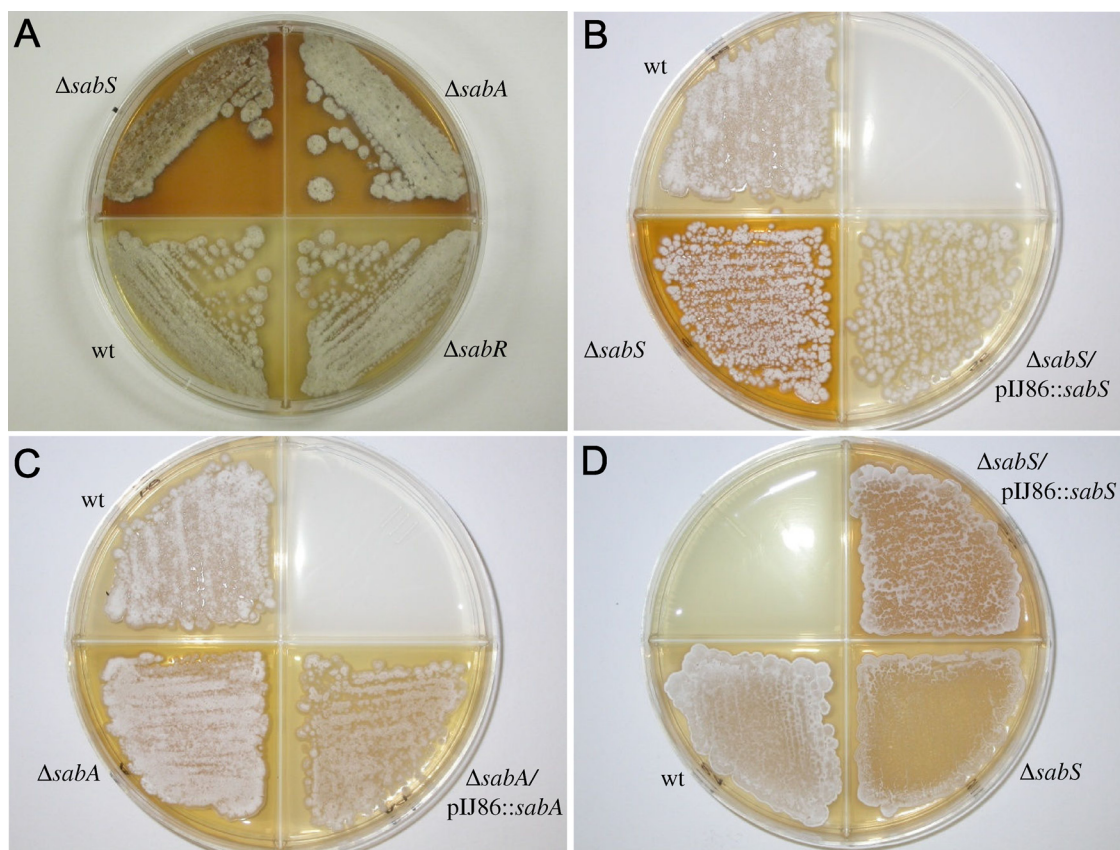


FIG. 4. Phenotypic properties of *sabR*, *sabA*, and *sabS* mutants. (A) Pigment production characteristics of *sabR*, *sabA*, and *sabS* deletion mutants on SGM. wt, wild-type strain *S. acidiscabies* 84.104. (B) Complementation of *sabS* deletion mutant on SGM with plasmid pIJ86::*sabS*. (C) Complementation of *sabA* deletion mutant on SGM with plasmid pIJ86::*sabA*. (D) Complementation of conditional morphological defect of *sabS* mutant on ISP2 medium with plasmid pIJ86::*sabS*.

Lower increases were seen with the addition of 10, 20, and 30 mM $MgCl_2$, and no difference in transconjugant numbers was observed between 40 mM and 50 mM $MgCl_2$ amendments (data not shown). Similar transconjugant frequencies were obtained using either *E. coli* conjugal donor strain S17-1 or ET12567(pUZ8002).

The wild-type strain and the $\Delta sabR$, $\Delta sabA$, and $\Delta sabS$ mutants were grown on ISP2 medium, SGM, and oatmeal agar medium to identify differences in colony morphology and/or in the appearance of pigmented secondary metabolites. SGM supports production of the yellow pigmented angucyclinone polyketide WS5995B (21), whereas oatmeal-based medium supports production of high levels of thaxtomins in *S. acidiscabies* (28). After growth in oatmeal broth medium, no differences in growth yields between the wild-type and mutant strains were observed (data not shown). Similarly, following extraction and silica gel and reverse-phase TLC analysis of oatmeal broth culture filtrates, no differences in thaxtomin production were seen between the wild-type and mutant strains (data not shown).

While no changes in thaxtomin production were seen between the wild type and the mutants, a comparison of the wild type and the $\Delta sabA$ and $\Delta sabS$ strains grown on solid SGM showed that these two mutants produced higher levels of a yellow diffusible pigment than the parent strain (Fig. 4A). No

significant differences in pigment production relative to the parent strain were observed in $\Delta sabR$ mutants on SGM or oatmeal agar medium (data not shown). To verify that the pigment overproduction phenotypes observed in *sabA* and *sabS* mutants were due to the mutant *sabA* and *sabS* alleles, the wild-type alleles of these genes were cloned into plasmid pIJ86 under the transcriptional control of the constitutive *ermE** promoter (1). Normal wild-type levels of pigment production were seen in mutant transconjugants carrying the plasmids, demonstrating that pigment production phenotypes were due to mutations in *sabA* and *sabS* (Fig. 4B and C).

In addition to differences in pigment production by *sabS* and *sabA* mutants, we also observed morphological differences in *sabS* mutants when the organism was cultured on ISP2 agar medium, a rich medium in comparison to mannitol-based SGM or oatmeal-based medium. Mutants grown on ISP2 medium largely failed to produce aerial hyphae from substrate mycelium, even after prolonged growth (Fig. 4D). Partial restoration of normal morphological development was observed in *sabS* mutants on ISP2 when they carried wild-type *sabS* on the multicopy plasmid pIJ86 (Fig. 4D). This effect was not observed when the *sabS* mutant was cultured on mannitol- or starch-based medium, such as SGM (Fig. 4B). While aerial hyphal development of *sabS* mutants was not impaired on SGM, we did observe a more compact, tighter colony morphol-

ogy of *sabS* mutants on SGM plates. Partial restoration of normal colony morphology was observed in *sabS* mutants on SGM when they carried wild-type *sabS* on the multicopy plasmid pIJ86 (Fig. 4B).

The conditional morphological defect observed on ISP2 medium is reminiscent of effects seen in, e.g., *S. coelicolor bldA* mutants or other strains (e.g., *adpA* mutants) with defects in genes containing UUA codons when cultured on rich, high-osmolarity medium such as R2YE (24, 27, 33). Morphological defects can be suppressed in such mutants when cells are cultured on mannitol-based medium. The *bldA* gene encodes tRNA^{leu} and recognizes rare UUA codons in high-GC content *Streptomyces* mRNA transcripts. Numerous genes containing TTA codons have been identified, and many of these are genes involved in morphogenesis or secondary metabolism (5). Notably, the *sabS* gene sequence possesses a TTA leucine codon in the 5' end of the gene. The codon sequence occurs at either nucleotides 13 to 15 (the 5th triplet codon) or at nucleotides 52 to 54 (the 18th triplet codon), depending on which translation start codon is used (see also Fig. 6A). Based on alignments with other GBL receptor homologs, the amino acid position occupied by leucine in SabS resides in the N terminus of the protein in a region preceding the DNA binding HTH domain. Further, this amino acid position is usually occupied by glutamic acid (12 out of 18 proteins examined); there are no other occurrences of leucine at this position in accessible GBL receptor sequences we examined. Although specific target genes subject to regulation by SabS have not yet been identified, these results suggest that SabS could function as a transcriptional regulator of genes related to morphological development. These studies are currently under way.

To more thoroughly investigate the nature of the diffusible yellow pigment compound produced in excess in *sabS* and *sabA* mutants, the mutants and wild-type strains were grown in liquid SGM, and culture filtrates were extracted using solid-phase methods. Extracts were analyzed using silica gel TLC and compared with preparations of pure WS5995B. Extracts of the *ΔsabS* and *ΔsabA* culture filtrates contained higher levels of a yellow pigment that was also present in the wild-type culture filtrate extracts, indicating overproduction of a metabolite in the mutant strains. The compound also displayed chromatographic properties on TLC plates similar to pure WS5995B. The yellow compound was recovered from preparative silica gel medium, and absorbance scans revealed that the material exhibited spectroscopic properties identical to those of pure WS5995B (data not shown). The properties of silica gel-purified compound as determined by analytical high-performance liquid chromatography were also identical to those of pure WS5995B (data not shown). While higher levels of WS5995B were found in both *ΔsabS* and *ΔsabA* mutants, the amounts of WS5995B produced by the *ΔsabS* mutant were substantially higher than in the *ΔsabA* mutant.

In order to investigate how the *sabS* mutation affected WS5995B production rates, growth of the wild-type and *ΔsabS* strains was measured over the course of WS5995B production. Detailed metabolite kinetics studies were not done with the *sabA* mutant. For studies of metabolite yields relative to growth in *sabS* mutants, it was necessary to follow growth kinetics by cell mass determinations rather than by using absorbance spectroscopy since growth of the organisms in SGM

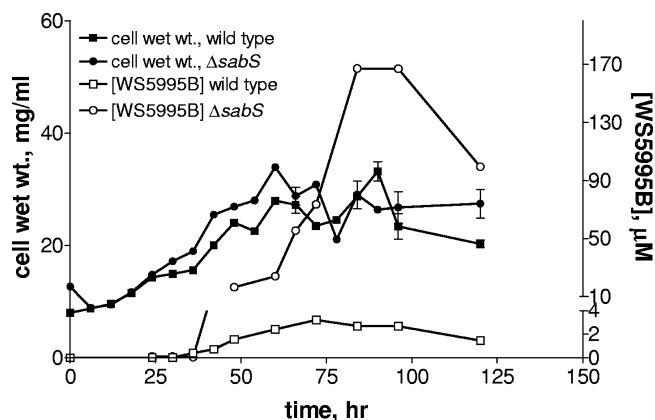


FIG. 5. Growth and WS5995B production characteristics of *S. acidiscabies* 84.104 wild type and a *ΔsabS* deletion mutant. Cell weight and WS5995B production of the wild type and the *ΔsabS* mutant are given, as indicated on the figure. Given large differences in WS5995B production for the wild-type and mutant strains, note that the right-hand y axis is segmented to allow inspection of WS5995B production rates for both strains. Over the 12-h interval from 36 h to 48 h, the rate of WS5995B production was $1.36 \mu\text{M} \cdot \text{h}^{-1}$.

is not dispersed sufficiently enough to allow accurate analysis using spectroscopic methods. Growth kinetics of the wild-type and GBL receptor mutant *ΔsabS* were similar (Fig. 5). Trace amounts of WS5995B were detectable in extracts from both strains within 24 h. While the onset of WS5995B production began at approximately the same time for both strains, notable differences in the patterns and rates of production of WS5995B were seen between the wild-type and mutant strains. Production rates of WS5995B by *ΔsabS* varied over a 72-h time course, whereas production by the wild-type strain remained constant. After 36 h, a rapid increase in the rate of WS5995B production was observed in the mutant relative to the wild-type strain ($1.36 \mu\text{M} \cdot \text{h}^{-1}$ for *ΔsabS* versus $0.1 \mu\text{M} \cdot \text{h}^{-1}$ for the wild type). At 48 h, the rate of production in *ΔsabS* decreased to that of the wild-type strain. At 60 h, WS5995B production increased again to $6 \mu\text{M} \cdot \text{h}^{-1}$. After about 72 h, WS5995B production in both strains reached plateau levels and began to decline soon thereafter.

SabR binds an ARE sequence element which overlaps the *sabS* translation initiation site. In order to explore the biochemical properties of the GBL receptors SabR and SabS, the respective ORFs were amplified separately and cloned into the plasmid expression vector pET26b. The proteins were expressed and purified as recombinant derivatives carrying C-terminal hexahistidine sequences. Following the preparation of soluble extracts from IPTG-induced *E. coli* cultures, the proteins were purified using standard immobilized metal affinity chromatography methods. Both proteins were recovered from columns in elution buffer containing 300 mM imidazole. Fractions were analyzed using sodium dodecyl sulfate-PAGE, and appropriate fractions were pooled and used for mobility shift assays.

We were interested in identifying ARE DNA sequences within the *sabRAS* region which were recognized by SabR and/or SabS. Using pattern search tools (38), we identified two sites within or in the proximity of the *sabRA* intergenic region with weak but significant similarity to previously characterized

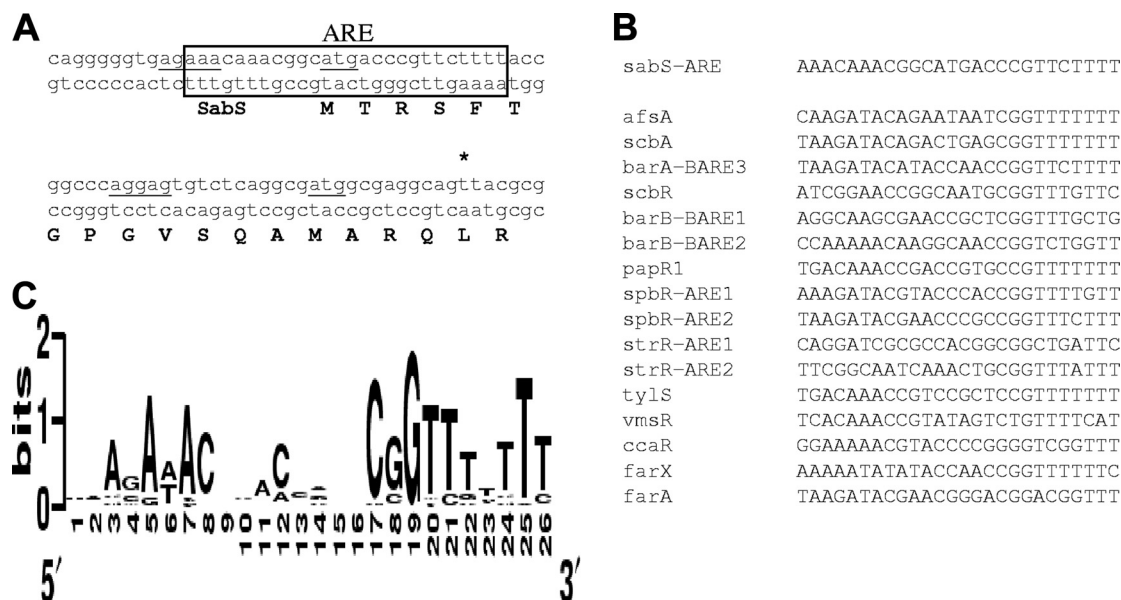


FIG. 6. *S. acidiscabies* 84.104 ARE sequence and ARE consensus. (A) Position of the ARE (boxed) relative to possible SabS translation initiation sites and the upstream region of *sabS*. Double-stranded DNA sequence is shown. Two possible alternative start codons and Shine-Dalgarno sequences are underlined, and the N terminus of the deduced SabS peptide sequence is shown below the DNA, with leucine specified by a TTA codon indicated by an asterisk. (B) Nucleotide sequence alignment of the predicted ARE sequence from *S. acidiscabies* (sabS-ARE) with previously characterized *Streptomyces* ARE boxes. (C) Sequence logo illustrating conservation of bases within aligned ARE sequences.

ARE sequences in other streptomycetes. These sites were found centered at -12 with respect to the *sabR* translation start and at $+14$ with respect to the *sabA* translation start (data not shown). A radiolabeled 294-bp PCR product was generated which encompassed the *sabRA* intergenic region and 5' ends of both genes, including predicted ARE sites. This labeled fragment was used in gel mobility shift assays to detect binding by SabR and/or SabS. Numerous reaction conditions were tested, and no specific binding activity was observed for either SabR or SabS, indicating that no ARE sequences are present in the *sabRA* intergenic region (not shown).

Pattern searching was also carried out to identify potential ARE sequences in regions upstream of *sabS*. Results of this analysis revealed one sequence with very strong similarity to previously characterized streptomycete ARE sequences, located in the upstream region of *sabS* translation initiation site (Fig. 6). A 209-bp radiolabeled PCR amplification product encompassing this ARE was used for gel mobility shift analysis to detect DNA binding activity with SabS and SabR. No DNA binding activity was detected with SabS in mobility shift assays under various conditions using this DNA substrate. The appearance of a single shifted complex was detected with SabR, however; results of these mobility shift experiments are shown in Fig. 7. A shifted complex using the DNA fragment encompassing the ARE sequence within the *sabS* region was observed at SabR concentrations as low as 2 nM. In order to verify specific SabR binding to the ARE sequence within the 209-bp radiolabeled fragment, mobility shift assays were done with the inclusion of unlabeled duplex ARE competitor DNA. DNA-SabR complex dissociation occurred with added competitor (Fig. 7, lane 8). An apparent dissociation constant, K_D , of 14 nM was calculated from fractional binding plots of imaged mobility shift data. These data demonstrate that SabR specif-

ically binds an AT-rich ARE box which covers the upstream region of *sabS*.

Given that SabR interacts with an ARE sequence which encompasses one of two possible *sabS* translation initiation codons, we propose that *sabS* translation initiation most likely occurs at the second initiation codon, with the ARE centered at -38 with respect to translation initiation. Other lines of evidence suggest *sabS* is translated from this second initiation codon, resulting in production of the smaller SabS protein. Namely, translation from the 5'-most initiation codon produces an N-terminal sequence not seen in other GBL receptors, based on protein alignments (data not shown). Also, as SabR represses *sabS* expression (see below), the transcription level control of *sabS* expression could most easily be explained by binding of SabR to regions upstream of *sabS* translation initiation. This region also includes sequences with high prob-

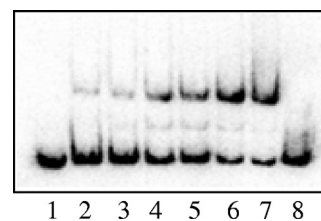


FIG. 7. SabR-ARE DNA mobility shift assay. A ^{32}P -labeled 209-bp DNA fragment encompassing the ARE box was incubated with various amounts of recombinant SabR, and binding reaction products were electrophoresed through a 5% native PAGE gel in $0.5\times$ Tris-borate-EDTA buffer. Lane 1, no SabR; lane 2, 1.5 nM SabR; lane 3, 3 nM SabR; lane 4, 6 nM SabR; lane 5, 12 nM SabR; lane 6, 18 nM SabR; lane 7, 24 nM SabR; lane 8, 24 nM SabR plus 150 nM unlabeled 34-bp ARE duplex competitor DNA.

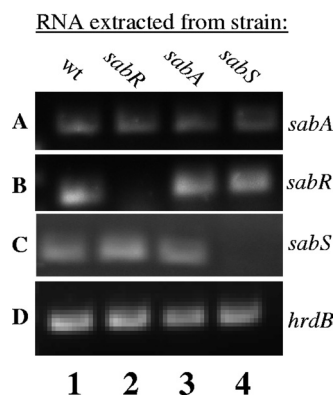


FIG. 8. Reverse transcriptase PCR analysis of *sabRAS* gene expression. Strains used for RNA extraction are given across four vertical columns, as indicated at top; PCR amplicons analyzed from cDNA samples are given over four horizontal rows, as indicated at right. *hrdB* represents a principal vegetative sigma factor. No bands are seen for a *sabR* amplicon from *sabR* RNA samples or for a *sabS* amplicon from *sabS* RNA samples because amplicon regions were removed in construction of these deletion mutants. The *sabA* amplicon is seen from *sabA* RNA samples because the amplicon region was retained in the creation of a *sabA* deletion strain.

abilities of promoter elements (data not shown). These areas of inquiry are currently being investigated.

***sabS* expression is elevated in *sabR* mutants.** In order to test how GBL genes might regulate one another, RNA samples were extracted from GBL mutants and wild-type strains. Reverse transcriptase was used along with the appropriate reverse oligonucleotide primers for the synthesis of cDNA from mRNA templates. cDNA products were then used for PCR amplification of *sabR*, *sabA*, and *sabS* amplicons to measure relative levels of these genes. Results of these assays are shown in Fig. 8. Compared with RNA samples from wild-type cultures, no significant differences were found in expression levels of *sabA* among *sabA*, *sabR*, or *sabS* mutants (Fig. 8, row A). In all strains *sabA* expression is lower than the levels of *sabR*, *sabS*, and *hrdB* expression observed in wild-type and mutant strains. The expression of GBL synthase genes has been shown to be lower than expression levels of their cognate receptors in other streptomycetes. For example, *barX* expression in *S. virginiae* is notably lower than the expression of the cognate receptor gene *barA* (20).

No amplification product is seen for the *sabR* gene from *sabR* mutant RNA samples (Fig. 8, row A, lane 2). This is because the *sabR* amplicon sequence resides within the region of *sabR* that was removed during construction of the *sabR* deletion mutant. Thus, we were unable in these experiments to determine whether *sabR* expression was altered in *sabR* mutants. We could conclude from our results, however, that there were no significant differences in *sabR* expression between *sabA*, *sabS*, and the wild-type strain (Fig. 8 row B, lanes 1, 3, and 4). The failure of SabS to bind sequences in the upstream region of *sabR* and consequently repress *sabR* expression is consistent with this finding.

The *sabS* amplicon sequence also occurs within a region of *sabS* which was removed in construction of the *sabS* deletion mutant; we were therefore unable to assess effects of the *sabS* mutation on *sabS* expression levels (Fig. 8, row C, lane 4).

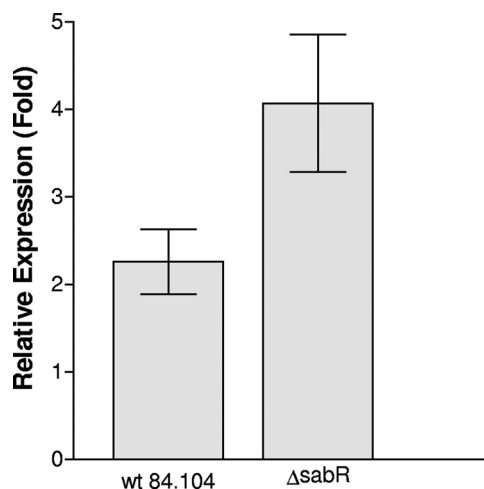


FIG. 9. qRT-PCR analysis of *sabS* expression in wild-type 84.104 and *sabR* mutants. Graph shows average relative increase in *sabS* expression in the wild type (wt) and *sabR* deletion mutant from three independent experiments. Expression differences were computed using the $\Delta\Delta C_T$ method. Significance was calculated by analysis of variance, and error bars represent standard error.

However, modest but reproducibly elevated levels of *sabS* were detected in *sabR* mutants compared with the wild-type strain; levels of PCR product obtained from amplification of cDNA from *sabR* strains were higher than those of products obtained from amplification of cDNA from the wild-type strain (Fig. 8, row C, compare lanes 1 and 2). Also, *sabS* levels in *sabA* mutants and wild-type strains were similar (Fig. 8, row C, lanes 1 and 3).

In order to confirm the RT-PCR results observed for *sabS* expression in wild-type and *sabR* mutant strains, qRT-PCR assays were performed. The results of these experiments show that *sabS* expression is elevated in *sabR* mutants, providing supportive evidence for the negative regulatory role of SabR in *sabS* expression (Fig. 9). Given that our mRNA samples were obtained from mid-exponential-phase cultures, we suspect that GBL was already present in the cultures. If, as we predict, SabR binds GBL and GBL binding occurs at extremely low concentrations (15), binary SabR/GBL complexes have probably already begun to dissociate from *sabS* promoter, resulting in the appearance of *sabS* transcripts in the wild-type RNA samples. We therefore suspect that the differences in *sabS* expression between the wild type and *sabR* mutants would be even greater with RNA samples recovered from the organisms at earlier time points.

Taken together, the results we describe in this experimental study suggest a role for GBL signaling gene homologs *sabRAS* in regulating both production of the aromatic polyketide WS5995B and morphogenesis in *S. acidiscabies*. Deletion of *sabS* results in overproduction of WS5995B, which could most easily be explained by postulating that SabS functions as a negative regulator of WS5995B polyketide synthase (PKS) gene expression. Conversely, the conditional defect in morphological development observed with *sabS* mutants grown on rich medium suggests that SabS acts to positively regulate morphogenesis. Given this dual role proposed for SabS, it is plausible that SabS functions in a manner similar to that of other tran-

scription factors which function as intermediates in the processing of GBL signaling inputs to multiple outputs. For example, in *Streptomyces pristinaespiralis* the GBL receptor SpbR controls pristinamycin production through binding at the promoter region of the pristinamycin pathway-specific activator *papRI*. Additionally, SpbR plays a role in morphological development (10). It is likely that SabS responds to a ligand and that target gene expression is mediated through this interaction. While SabS does not bind ARE sequences in the *sabRAS* region, it is plausible that, given its similarity to other GBL receptors, SabS binds ARE sequences elsewhere in the genome in a manner which would result in negative regulation of expression of a type II PKS gene cluster encoding enzymes required for WS5995B biosynthesis and in positive regulation of genes involved in morphogenesis. Whether such control phenomena would be exerted directly or indirectly, through pathway-specific promoter interactions such as occurs at, e.g., *papRI* is unknown. We are currently developing methods to identify DNA binding sites for SabS to elucidate the position of SabS within a regulatory network which would govern secondary metabolism and morphological development. Using a strategy similar to the one we used for the identification of GBL receptor genes (48), we have also identified type II PKS gene sequences from *S. acidiscabies* genomic DNA which may encode WS5995B PKS and pathway activators regulated by SabS-DNA interactions.

While it is tempting to speculate that SabS and SabR bind GBL compounds produced by *S. acidiscabies* since they are clearly homologous with other characterized GBL receptor proteins and since they lie immediately adjacent to the GBL synthase homolog *sabA*, we have no direct evidence at this time that either protein binds GBL compounds. It is for this reason that we refer to SabS and SabR as GBL receptor homologs, pending biochemical verification of GBL binding properties of these proteins. There are instances of GBL receptor homologs which do not appear to bind GBLs. For example, regulation of alpomycin biosynthesis in *Streptomyces ambofaciens* occurs through interactions between the GBL receptor homolog AlpZ and a ligand exhibiting chemical properties inconsistent with those of GBL compounds (4). Also, it has been reported that the *S. virginiae* GBL receptor BarB does not exhibit GBL binding activity (30). Moreover, based on phylogenetic arguments, it has been proposed that "GBL receptors" predate GBL synthases and initially functioned as DNA binding proteins prior to acquiring GBL binding properties (34). Thus, SabR and/or SabS may bind ligands other than GBL compounds.

As GBL compounds are typically produced in minute quantities and are recovered through extraction into organic solvent, large volumes of culture (≥ 400 liter) and solvent are required for extraction to obtain quantities of material sufficient for absolute structural characterization of GBLs from *Streptomyces* (see, for example, reference 43). For these technical and economic reasons, we have not approached the question of a GBL structure from *S. acidiscabies*. However, using electrospray ionization mass spectrometry methods with culture extracts as described by Yang et al. (49), we have detected fragment ions bound by our purified GBL receptors with masses consistent with molecules possessing lactone functional groups such as those found in GBL compounds (data not

shown). The proximity of *sabA* to both *sabR* and *sabS* along with the conservation of residues in SabA predicted to be required for GBL synthase activity (19) invites the speculation that *sabA* encodes a GBL synthase. Technical challenges exist in the biochemical characterization of SabA. For example, since we do not have a GBL structure from our organism, we lack information regarding SabA enzymatic substrates.

Nonetheless, we find changes in WS5995B production and in morphological properties in *S. acidiscabies* GBL signaling pathway homolog mutants consistent with disruption of an autoregulatory GBL-like pathway. Let us assume, for example that *sabA* encodes a GBL synthase which produces a GBL ligand that, in turn, binds the divergently expressed GBL receptor SabR. Then, elevated production of WS5995B observed in *sabA* mutants could be attributed to SabR-mediated repression of *sabS*, resulting in derepression of WS5995B PKS expression. The observation that *sabR* mutants appear phenotypically similar to the wild-type strain can be explained by assuming that if SabR represses *sabS* expression and if SabS also binds GBL produced by SabA, the normal course of threshold accumulation of GBL produced by SabA would still result in dissociation of SabS from target regulatory sequences. Thus, *sabS* repression of PKS gene expression is still ultimately subject to control by GBL binding so that SabS can only repress PKS expression until GBL accumulates, and at that point one would observe nearly wild-type levels of WS5995B, even in a *sabR* mutant, since it would still synthesize GBL.

The significance of the TTA leucine codon in *sabS* is not known. Recent bioinformatics analyses of four sequenced *Streptomyces* genomes shows that TTA-containing genes are often associated with secondary metabolite biosynthetic gene clusters, particularly in those genes encoding likely regulatory functions (5). While the genome of *S. acidiscabies* has not been sequenced, we assume that UUA codons are recognized by a *bldA*-type tRNA, as seen in other streptomycetes (24, 26, 46, 47). Our RT-PCR data suggest that *sabS* expression is elevated in *sabR* mutants. If SabR represses *sabS* expression in the absence of a ligand (e.g., a GBL) and if the intracellular accumulation of ligand results in derepression of *sabS* expression, we suspect that translation of the GBL receptor SabS would be dependent on *bldA* tRNA.

The AdpA transcriptional regulator is encoded by a UUA-containing gene; it is present in all *Streptomyces* genomes sequenced to date and has been characterized in both *S. griseus* and *S. coelicolor*. AdpA plays a central role in morphogenesis and secondary metabolism in both organisms, and the gene possesses UUA codons in both organisms. Yet only the *S. griseus* *adpA* gene appears to function in a GBL-dependent regulatory cascade (6, 36, 44). AdpA-dependent expression of target genes in *S. griseus* occurs following derepression of *adpA* expression as a result of dissociation of A factor-ArpA complexes from *adpA* promoter. Given the central regulatory role of AdpA, it is possible that SabS could function downstream of AdpA in the *bldA*-dependent regulation of morphogenesis and WS5995B biosynthesis. Future research will be directed toward further characterization of the network governing secondary metabolism and morphogenesis in this organism.

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