Pleiotropic Functions of a Streptomyces pristinae spiralis
Autoregulator Receptor in Development, Antibiotic Biosynthesis,
and Expression of a Superoxide Dismutase

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Received for publication, February 5, 2001, and in revised form, September 5, 2001
Published, JBC Papers in Press, September 13, 2001, DOI 10.1074/jbc.M101119200

In Streptomyces, a family of related butyrolactones and their corresponding receptor proteins serve as quorum-sensing systems that can activate morphological development and antibiotic biosynthesis. Streptomyces pristinae spiralis contains a gene cluster encoding enzymes and regulatory proteins for the biosynthesis of pristinamycin, a clinically important streptogramin antibiotic complex. One of these proteins, PapR1, belongs to a well known family of Streptomyces antibiotic regulatory proteins. Gel shift assays using crude cytoplasmic extracts detected SpbR, a developmentally regulated protein that bound to the papR1 promoter. SpbR was purified, and its gene was cloned using reverse genetics. spbR encoded a 25-kDa protein similar to Streptomyces autoregulatory proteins of the butyrolactone receptor family, including scbR from Streptomyces coelicolor. In Escherichia coli, purified SpbR and ScbR produced bound sequences immediately upstream of papR1, spbR, and scbR. SpbR DNA-binding activity was inhibited by an extracellular metabolite with chromatographic properties similar to those of the well known γ-butyrolactone signaling compounds. DNase I protection assays mapped the SpbR-binding site in the papR1 promoter to a sequence homologous to other known butyrolactone autoregulatory elements. A nucleotide data base search showed that these binding motifs were primarily located upstream of genes encoding Streptomyces antibiotic regulatory proteins and butyrolactone receptors in various Streptomyces species. Disruption of the spbR gene in S. pristinae spiralis resulted in severe defects in growth, morphological differentiation, pristinamycin biosynthesis, and expression of a secreted superoxide dismutase.

Diffusible pheromones often coordinate expression of specific genetic programs within a population of bacteria as they reach high cell density. Pioneering studies leading to the discovery of γ-butyrolactone signaling molecules were made in Streptomyces, Gram-positive filamentous bacteria that include many important antibiotics. Since these studies, γ-butyrolactones have often been found to have antibiotic activity. Khokhlov et al. (1, 2) demonstrated that in Streptomyces griseus, these developmental programs could be coordinated by nanomolar concentrations of a molecule they identified as a γ-butyrolactone and named “A-Factor.” The chemical structures of many species-specific Streptomyces butyrolactones have since been determined, along with a growing family of putative receptor proteins (3–7). To facilitate subsequent discussions of the γ-butyrolactone signaling system, its components and their functions are diagrammed in Fig. 1.

Many γ-butyrolactone receptors (GABR) bind to a conserved nucleotide motif (autoregulatory element) (ARE) and thus act as repressors of transcription (8). The butyrolactones that accumulate in culture media are thought to act as quorum signaling molecules by releasing their corresponding GABR proteins from operator sites, thus activating gene expression (6).

Developmental systems under butyrolactone control have been best characterized in S. griseus and Streptomyces vir giniae. Studies of Horinouchi, Beppu, and co-workers (9) support a model describing how A-Factor and its receptor in S. griseus, ArpA, mediate pleiotropic effects on development. Binding of A-Factor to ArpA derepresses expression of a transcriptional activator, AdpA (10). AdpA promotes expression of sirR, the activator of streptomycin biosynthetic genes, and other unknown genes that control aerial mycelium formation. In S. vir giniae, butyrolactones and a corresponding receptor (BarA) take part in regulating synthesis of a streptogramin complex called virginiamycin (11) via unknown regulatory pathways. Related Streptomyces antibiotic regulatory proteins (SARPs) commonly activate expression of biosynthetic gene clusters. Thus, SARPs are potentially the ultimate target for some quorum-sensing signaling pathways that switch on antibiotic biosynthesis (12).

Gram-negative bacteria employ quorum-sensing systems based on homoserine lactones, structurally related to γ-butyrolactones, to control a diverse array of density-dependent phenotypes (13, 14). In Pseudomonas, quorum-sensing systems...
control synthesis of virulence factors as well as enzymes such as catalases that detoxify reactive oxygen species (ROS) and superoxide dismutase (15).

Superoxide dismutases are ubiquitous parts of cellular defenses against oxidative stress that catalyze dismutation of the toxic superoxide anion into hydrogen peroxide (H2O2), thus preventing the spontaneous formation of more toxic forms of ROS by the Haber-Weiss reaction. The two classes of superoxide dismutase found as the major protein in the medium.

ROS may come from endogenous metabolism or exogenous sources. Primary metabolic conversions that generate ROS are largely limited to flavin and flavoproteins that activate molecular oxygen (17). Such monooxygenases often participate in the respiratory chain within the cytoplasmic membrane. In contrast, pathogenic bacteria probably employ superoxide dismutase to defend themselves against external ROS they may encounter as part of the host antimicrobial response. The SodF released by Mycobacterium tuberculosis is thought to protect the organism from oxidative attack by macrophages (18).

Our studies involved S. pristinae spiralis, a saprophytic soil organism that produces pristinamycin, a clinically important streptogramin antibiotic complex. Like other streptograms, it is a mixture of compounds based on two structurally dissimilar synergistic antibiotics. The streptogramin B component, pristinamycin A (P), is a cyclic hexadepsipeptide; the streptogramin A compound, pristinamycin II (PII), is a polyunsaturated cyclic peptolide (19).

PI and PII biosynthetic genes are clustered together with papR1 (putative regulator of pristinamycin antibiotic production), the SARP gene described here. We purified SpbR (S. pristinae spiralis butyrolactone-responsive transcriptional repressor), a GABR protein that bound to a site upstream of the papR1 promoter, and showed that the corresponding gene was required not only for colonial development and antibiotic biosynthesis, but also for expression of a leaderless superoxide dismutase found as the major protein in the medium.

**Experimental Procedures**

The bacterial strains included E. coli SG13609, XL1-Blue, and M15; S. pristinae spiralis NRRL2958; Streptomyces coelicolor MT1110; and Bacillus subtilis ATCC6633. pUC18, pUC21, and the expression system pDS56/RRBSII were used as E. coli vectors. Restriction enzymes, T4 DNA ligase, and T4 DNA polymerase were purchased from New England Biolabs Inc. Techniques for handling Streptomyces have been described (20).

**Transformation**

Streptomyces protoplasts were transformed, spread on R2YE medium, and allowed to regenerate for 20 h at 30 °C (20). Transformants were selected by overlaying the plates with 1 ml of aqueous apramycin (1 mg) or thiostrepton (0.3 mg). To enhance integration into the chromosome via homologous recombination, plasmids were alkali-denatured before protoplast transformation (21). E. coli cells were transformed by calcium shock or electroporation (22).

**Growth Media**

HT7T contained (per liter): white dextrin, 10 g; NZ amine-A, 2 g; Lab Lemco beef powder, 1 g; yeast extract, 1 g; and 1 ml of trace elements stock solution (CaCl2·2H2O, 11 g; FeCl3·6H2O, 7 g; MnCl2·4H2O, 2 g; ZnSO4·7H2O, 2 g; CuSO4·5H2O, 0.4 g; CoCl2·6H2O, 0.4 g; 45 g/L EDTA-Na2·9H2O, in 11 of ddH2O pH 7.4).

NE solid medium contained 10 g/liter glucose, 2 g/liter yeast extract (Difco), 1 g/liter Lab Lemco powder, and 15 g/liter agar (Difco) (pH 7.0). Minimal inoculum medium contained 20 g/liter succharose, 5 g/liter (NH4)2SO4, 0.75 g/liter KH2PO4, 0.5 g/liter MgSO4·7H2O, 1 ml of trace element stock, and 40 g/liter MOPS (pH 6.8). Minimal production medium contained 40 g/liter glucose, 13 g/liter l-glutamate, 1.2 g/liter KH2PO4, 0.3 g/liter MgSO4·7H2O, and 1 ml of trace elements stock solution (pH 6.8). Mannitol soya medium contained 20 g/liter mannitol, 20 g/liter soya meal, and 20 g/L Difco agar.

**Growth Conditions**

S. pristinae spiralis NRRL2958 spores (5 × 108) grown on HT7T medium were inoculated in a 250-ml baffled flask containing 100 ml of minimal inoculum medium. After incubation on a rotary shaker at 200 rpm for 26 h at 30 °C, the culture served as inoculum for a 2-l pilot fermenter containing minimal inoculum medium (pH 6.8). The fermentation was carried out at 28 °C with constant aeration. For strains containing plasmid pNL5 or pIJ904, the media were supplemented with thiostrepton (3 μg/ml).

**Gel Retardation Assay for DNA-binding Proteins**

DNA fragments were end-labeled by filling in with Klenow fragment or fully labeled by PCR in the presence of [α-32P]dATP. This generated probes of different specific activities for use in gel retardation assays.

Crude or semipurified DNA-binding proteins (5 μg) were incubated with radiolabeled DNA fragment (0.006 pmol of the filled-in probe and 0.06 pmol of the PCR-generated probe) in the presence of 1–5 μg of competitor DNA (poly[dI-dC]/[dI-dC]; Amersham Pharmacia Biotech) in 20 μl of tris buffer containing additives (TA; 10 mM Tris (pH 7.5), 10 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Triton X-100, and 10% glycerol) supplemented with 250 mM NaCl. After incubation, the mixture was resolved on 5% polyacrylamide gels in 90 mM Tris, 90 mM borate, and 2 mM EDTA (pH 8), run at room temperature and constant voltage (7 V cm⁻¹) for 2 h. Gels were dried, and radioactive bands were recorded by autoradiography or on a Phosphorimager.

A 322-bp fragment of S. pristinae spiralis DNA (nucleotides 113–435; GenBank®/EBI Data bank accession number AY026762) including the region upstream of papR1 was amplified by PCR (forward primer, CCCAAAGTCTGAACAGCGCTCTACCAA; and reverse primer, CGG GATCCATGGGCTTGTGCTT). and subcloned into HindIII/BamHI-cleaved plasmid pUC18 (pMF1).

For the PapR-ARE (where “P” is promoter) and PapR-ARE2 regions, the region upstream of spbR corresponding to nucleotides 40–424 (accession number AY026762) was PCR-amplified (forward primer, GGTTCGCTGTTGCAGTTAGGATG; and reverse primer, CGGC GCGTTCTACAGGGCCGCTCCTGCGCG). The two ARE sequences encoded by this fragment were separated by cleavage at an NdeI site (position 162). The intergenic region between adhC and mutT was PCR-amplified (forward primer, CTGTCGGGGGCTGGCTGGCG; and reverse primer, GCCTCGAGACCCAGAGGTTG CCT) to generate the Padh fragment (nucleotides 1351–1516; accession number AL357432). The region upstream of schR was PCR-amplified (forward primer, AAAA AACTCTGTTTGCCCAGATGT; and reverse primer, GGCATC GCCGTTCTACGGTTGCG) to generate the PschR fragment (nucleotides 2896–3056; accession number AJ007731).

**FIG. 1. The butyrolactone signaling cascade.** γ-Butyrolactones (●) and their regulators (GABR) can act as transcriptional repressors by binding to AREs. SARP proteins are needed for the expression of most antibiotic biosynthetic pathways. Studies of spbR. and the papR1 promoter established a robust consensus sequence for GABR binding and that GABR proteins bind directly SARP promoters as well as undefined promoters needed for growth, development, and expression of the central oxidative stress adaptive enzyme superoxide dismutase (SOD) SodF.
**SpbR Purification**

**Preparation of Crude Extracts**—Stationary phase mycelia grown in HT7T medium were harvested by centrifugation, washed twice with 250 ml of TA buffer supplemented with 10 mM NaCl (TAN buffer), and stored at −80 °C. All steps of the purification were carried out at 4 °C. The mycelia (∼100 g), harvested from 15-liter cultures, were disrupted by bead beating in a 200 ml of TA buffer supplemented with 100 mM NaCl. Protease inhibitors (benzamidine, pepstatin, and leupeptin; 130 g) were added prior to sonication. After sonication, phenylmethylsulfonyl fluoride (10 mM) was added. Cell debris was removed by centrifugation for 45 min at 9000 rpm in a Sorvall GSA rotor (13,000 × g).

**Ammonium Sulfate Precipitation**—Ammonium sulfate (Schwarz/Mann) was slowly added to the cooled protein extract to a final concentration of 28% (w/v). The extract was centrifuged at 13,000 × g for 60 min at 4 °C in a Sorvall GSA rotor. Soluble proteins were precipitated using ammonium sulfate (60% (w/v) final concentration; Sigma) and collected by centrifugation at 13,000 × g for 60 min in a Sorvall GSA rotor. The pellet was resuspended in 150 ml of TAN buffer, dialyzed against 10 liters of the same buffer, and then clarified by centrifugation for 30 min in a Sorvall SS34 rotor at 12,000 rpm (17,300 × g). The protein pellet was redissolved in TA buffer.

**DEAE Anion-exchange Chromatography**—The protein sample was then loaded onto a 150-ml DEAE-Sepharose column (XR 26/50, Amersham Pharmacia Biotech) equilibrated with TAN buffer. The column was washed with TAN buffer, and proteins were eluted in a 600-ml salt gradient of 10–450 mM NaCl in TA buffer. An aliquot (4 µl) of each 5-ml fraction was tested by gel mobility shift assays. The active fractions were pooled and supplemented with NaCl to make the conductivity equivalent to that of 100 mM NaCl in TA buffer.

**Heparin Chromatography**—Active DEAE fractions were loaded onto a 50-ml heparin-Sepharose CL-6B column (XR 26, Amersham Pharmacia Biotech). SpbR activity was detected in the early fractions of a 600-ml salt gradient of 10–450 mM NaCl in TA buffer. Fractograms were loaded on a 1-ml MonoQ column (HR5/5, Amersham Pharmacia Biotech). SpbR activity was detected in the early fractions of a 60-ml salt gradient containing the disrupted spbR gene and allowed it to be subcloned into pSET151 (25), a non-replicative plasmid that has the thiostrepton resistance marker. The construct (pNL6) was alkali-denatured and used to transform S. pristinaespiralis NRR72985 protoplasts (21). Among 90 apramycin-resistant transformant colonies, only one was thiostrepton-sensitive (spbR25).

To show by complementation in trans that the phenotypes observed were due to the disruption spbR gene, a plasmid containing only spbR and its promoter was constructed. The spbR gene was removed from pH1 by cleavage at BamHI (within insert)EcoRI (vector) sites and ligated with pUC21 cleaved by the same enzymes. The fragment containing spbR was excised by BamHI and BglII (pUC21-encoded) and cloned into pJB904 at the BamHI site (pNL5).

**Disruption of the spbR Gene**

A fragment containing the disrupted spbR gene and its flanking regions was cloned into a non-replicative plasmid (pSET151; construction described in the Fig. 2 legend) with the selectable thiostrepton resistance marker. This plasmid (pNL4) was used to transform S. pristinaespiralis. Among 100 apramycin-resistant transformants, only one was thiostrepton-sensitive. Southern hybridization (see Fig. 2B) showed that this clone (spbR25) contained the expected disruption of spbR resulting from a double crossover event. A low copy number plasmid containing the intact spbR gene (pNL5) (see Fig. 2A) was able to restore this activity in trans.

**Recombinant SpbR Protein Produced in E. coli**

The coding region of the spbR gene was amplified by PCR using oligonucleotides NeuNT (5′-ACAACATATGGCGCGGGCGGACAGGCGG-3′) and NeuCT (5′-GGTAAGCTTTGGTGGGGTGGGTCAGT-3′) and NeuCT (5′-GGTAAGCTTTGGTGGGGTGGGTCAGT-3′). The amplified fragment was inserted into the Ndel/HindIII sites of the expression plasmid pDS56 (phg2), and phg2 was used to transform E. coli M15 carrying a plasmid that supplies Lac repression (pREP4). This transformant was grown in LB medium supplemented with 100 µg/ml ampicillin and 25 µg/ml kanamycin. Synthesis of the protein was induced by the addition of 2 mM isopropyl-β-D-thiogalactopyranoside. Purification of the recombinant

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**Fig. 2. Construction of an spbR mutant, reduction of autoproteolytic cleavage, and N-terminal amino acid sequence determination.**

The schematics indicate the mutant (A) of the spbR gene (Δ), wild-type; (B) wt, wild-type strain; 1, wt-type strain; Δ, spbR25.
protein was done according to the procedure used for the isolation of SpbR from *Streptomyces* cell extracts, omitting the DNA affinity chromatography step.

**Isolation and Expression of the *scbR* Gene**

An *S. coelicolor* homolog of *scbR* was isolated from genomic DNA. A PCR fragment was amplified using degenerate primers based on conserved amino acid motifs within the DNA-binding domains of GABR genes *bara*, *arpA*, and *farA*: ALYFHFA (SCGAAAGTGGAAATTGSSGC) and AAAEVFDE (GCSCGSCGAGTSTTCGACGCA). This 150-bp fragment was used as a hybridization probe to clone a 5-kb region of the locus. The sequence of the *scbR* ORF was later found to be identical to that recorded by E. Takano (accession number AJ007731) and by the Sanger Center *S. coelicolor* Genome Sequence Project (accession number AL132824 http://www.sanger.ac.uk/Projects/S_coelicolor/). The *scbR* gene was subcloned as a 5-kb BclI fragment into the BamHI site of pUC18 (pMF10).

**Superoxide Dismutase Assays**

Crude cell extracts separated on SDS-polyacrylamide gels were transferred onto a nitrocellulose membrane and probed with rabbit anti-*M. tuberculosis* superoxide dismutase antibodies provided by M. A. Horwitz (UCLA) and then with swine peroxidase-conjugated anti-rabbit antibodies. Crude cell extracts were also separated electrophoretically on nondenaturing acrylamide gels, and superoxide dismutase was stained *in situ* (26).

**Southern Blot Hybridization**

Digested genomic DNAs were separated on 1% agarose gels, transferred by vacuum blotting to nitrocellulose membranes (Hybond N+) and probed by Southern hybridization under standard conditions (22) or under low stringency (0.2% SSC and 0.5% SDS at room temperature).

**Polymerase Chain Reaction**

PCRs were carried out using 100 pmol of primer, buffer supplied by PerkinElmer Life Sciences, and a Protocol Thermocycler (AMS Biotechnology). Standard hot-start PCR and touchdown PCR were performed in the presence of 10% Me2SO.

**DNase I Footprinting**

The *PpapR1* fragment was removed from pMF1 by *HindIII/SmaI* digestion and end-labeled by filling in the *HindIII* overhang with [α-32P]dATP. The probe was incubated with a saturating amount of purified SpbR (determined by gel retardation assay) in the presence of 4 μg of competitor DNA (poly[dI-dC]·(dI-dC)) and 0.5 units of DNase I. The digestion products were resolved on a 6% sequencing gel.

**Analysis of Secondary Metabolites**

Secondary metabolites in the media were extracted in ethyl acetate, resolved in *MeSO*, and assayed directly for antibiotic activity (pristinamycin) or separated by HPLC. The disc antibiotic assay utilized *B. subtilis* ATCC6633 growing on NE agar as an indicator lawn. To identify individual components of the pristinamycin complex, ethyl acetate extracts were applied to a reverse-phase column (μRPC C8/C18, Sc2,1/1.0 SMART; Amersham Pharmacia Biotech) in 0.1% trifluoroacetic acid and eluted in a linear gradient of H2O and acetonitrile. Fractions were assayed for compounds that inhibited SpbR DNA-binding activity.

**Protein Quantification**

Protein content was measured using a kit supplied by Bio-Rad with bovine serum albumin as a standard.

**RESULTS**

An SARP Gene Is Present in the Pristinamycin Biosynthetic Cluster—*papR1*, encoding a protein (284 amino acids) homologous to the SARP family, was identified in the course of sequencing the pristinamycin biosynthetic gene cluster (nucleotides 431–1283; accession number A37840). BLASTP protein data base searches showed that PapR1 has the highest similarity (75% identity) to TyJS and significant matches with a family of SARPs required for expression of various *Streptomyces* antibiotic biosynthetic gene clusters, including *DrrR1* (56% identity; daunomycin), *MtmR* (45% identity; mithramycin), and ActII-ORF4 (37% identity; actinorhodin). Other SARPs tested have proven to be essential for antibiotic biosynthesis; gene disruption of *papR1* reduced both PI and PII yields by only 30%. This may reflect the activity of a second SARP gene recently identified in the pristinamycin gene cluster.2 We assumed that these redundant (*papR2*) genes were involved in the control of pristinamycin biosynthesis, and studies of *papR1* might identify higher level regulators coordinating synthesis of PI and PII with developmental signals. Interestingly, visual inspection of the sequence upstream of the *papR1* transcriptional start site identified a potential ARE (see below).

**Pristinamycin Biosynthesis during Stationary Phase Is Associated with Alterations in the DNA-Binding Activity of a Putative Regulator of the *papR1* Promoter—**Samples of a growing culture (Fig. 3A) were assayed for pristinamycin biosynthesis as well as for proteins that potentially regulate the *papR1* promoter (*PpapR1*) (Fig. 3B). Bioassays showed that pristinamycin antibiotic activity accumulated in the medium beginning shortly after the maximum mycelial mass was attained (40–100 h) (Fig. 3A). Gel retardation assays using a fragment encoding the *papR1* promoter region (*PpapR1*) detected a potential GABR transcriptional regulatory protein (*SpbR*) in crude cytoplasmic extracts prepared from these cultures. *PpapR1* gel shift activity, not detected in growing cultures (Fig. 3B), was more pronounced in stationary phase cultures.
3B), increased shortly after the cultures entered stationary phase, coincident with the activation of pristinamycin biosynthesis (Fig. 3A).

Purification of SpbR from S. pristinae spiralis—SpbR was enriched from crude extract (7 g of protein) of stationary phase mycelia (100 g) using sequential DEAE, heparin, and MonoQ ion-exchange columns (see "Experimental Procedures"). The final step of the purification employed biotinylated PpapR1 fragment fixed to streptavidin-coated beads (23). The fact that SpbR remained fixed to the matrix after exposure to either nonspecific competitor DNA or high salt concentrations suggested specific interactions and allowed a 100-fold purification. SDS-polyacrylamide gel electrophoresis analysis (Fig. 4A) of the activity (Fig. 4B) specifically eluting at high salt concentrations (>250 mM) (lanes 6–9) suggested that it corresponded to a protein with an apparent mass of 28 kDa. This protein (3 μg, 93 pmol) was blotted onto a nitrocellulose filter, and its N-terminal sequence (determined by Edman degradation) was identical (90% identity) to bacterial MARQERAV. Four internal peptides generated by Staphylococcus aureus V8 endoproteinase had the following N-terminal sequences: LTVEQGAL, VADLY, DFSP, and VLAYEEAVRR.

Cloning and Sequencing of the S. pristinae spiralis spbR Gene—Oligonucleotides based on N-terminal (MARQERAV) and internal (LTVEQGAL) SpbR amino acid sequences were used to amplify the 5′-region of spbR, thereby facilitating the cloning and sequencing of the corresponding locus (described under "Experimental Procedures"). The locus was cloned on a 4.1-kb fragment in pUC18 (pHg1). Extracts of this strain of E. coli retarded migration of the PpapR1 fragment, whereas strains containing the vector alone were inactive.

DNA sequence analysis predicted SpbR to be a 228-amino acid protein (25.9 kDa) with similarity (40–60% identity) to bacterial...
transcriptional regulators belonging to the GABR family, including FarA, BarA, ArpA, JadR1, and TylP. SpbR is most similar to TylP in the *Streptomyces fradiae* tylosin gene cluster. Expression of *spbR* in *E. coli* and purification of recombinant SpbR (see "Experimental Procedures") provided definitive proof that SpbR is the *PpapR1*-binding protein. Recombinant SpbR had chromatographic characteristics (on DEAE, heparin, and Superdex 200 columns) similar to those of the purified native protein. Purified recombinant SpbR migrated as a single molecular species corresponding to ~50 kDa on a size-exclusion column (Superdex 200) (Fig. 5A). These data established the purity (>95%) of the recombinant protein and suggested that both native and recombinant SpbR formed dimers in solution. Finally, recombinant SpbR retarded migration of the *PpapR1* fragment in a manner indistinguishable from that detected in *S. pristinaespiralis* (Fig. 3B, lane 8). The binding curve determined an approximate *Kd* of 3 × 10^{-8} M (Fig. 5B) for the monomer or 1.5 × 10^{-8} M based on its apparent dimeric form (Fig. 5A).

A Butyrolactone-like Compound Inhibits SpbR Binding—An inhibitor of SpbR (purified recombinant protein) gel shift activity was detected in ethyl acetate extracts (note that ethyl acetate extracts amphipathic compounds such as PI, PII, and butyrolactones) of the culture medium either before (32 h) (Fig. 6A) or after (64 h) (Fig. 6B) antibiotic activity appeared in the medium. Although very little A_{215}-adsorbing material (Fig. 6A) was detected in the 32-h culture, fraction 21 inhibited formation of the SpbR-*PpapR1* complex. This activity was also present in late stationary phase cultures (Fig. 6B) and likewise eluted from the HPLC column in fraction 21. In both cases, the inhibition was concentration-dependent, suggesting specific inhibition as has been reported for other butyrolactone-binding proteins: FarA (27), BarA (27), and ArpA (28). Although molecules belonging to the pristinamycin complex (a mixture of compounds representing biosynthetic intermediates or derivatives of PI and PII) were also present in stationary phase cultures, formation of the SpbR-*PpapR1* complex (~0.1 μM) was not inhibited by molar excesses (~0.5 μM) of PI or PII. The *S. griseus* γ-butyrolactone (A-Factor) also eluted in this part of the HPLC gradient (fraction 22).

Gel retardation assays showed that several structurally related butenolides inhibited binding of SpbR (0.15 μM) to the...
PapR1 fragment (0.003 μM; data not shown), albeit at high concentrations. These low affinity ligands included the S. griseus A-Factor (10 μM) and Mp133 (1 μM), a butenolide from Streptomyces antibioticus (29). Other related lactones (y-valerolactone, 4-nonanolide, nonanal, and homoserine lactone) did not inhibit binding within these concentration ranges.

These results indicated that SpbR interacted most specifically with an S. pristinaespiralis ligand that was similar to Streptomyces butyrolactone quorum-sensing autoregulators.

**DNA-binding Motifs for Autoregulatory Proteins**—Purified recombinant SpbR protein protected a 31-bp sequence of the PapR1 fragment against DNase I digestion (Fig. 7A). This result showed that SpbR recognizes a sequence motif found upstream of SARP and GABR genes.

**Fig. 7.** SpbR recognizes a sequence motif found upstream of SARP and GABR genes. A, DNase I footprinting of an SpbR-binding site in the papR1 promoter region. A DNA fragment (nucleotides 113–435) encoding the nucleotide sequence upstream of papR1 was partially digested with DNase I in the presence or absence of sufficient purified recombinant SpbR to fully saturate its binding site (SpbR was titrated using the same DNA fragment and assayed by gel retardation). These digests were separated on a 6% acrylamide gel and compared with a sequencing ladder. The sequencing ladder was generated using a primer corresponding to the HindIII end of the probe fragment (AGCTTCGACACCGCTCTACCA). B, data base searches identify operator motifs belonging to the ARE family. A matrix of conserved nucleotide residues from seven ARE sequences that have been verified by DNase I footprinting or gel retardation (listed in the first sequence series) was compiled to search the Streptomyces sequences (Matrix 1) in the GenBank™/EBI Data Base using Mat-ind and Mat-Inspector (kindly provided by K. Quandt). This analysis identified six additional motifs that were all located upstream of genes belonging to the GABR or SARP family (second sequence series). These 13 sequences were used to compile Matrix 2, which identified the six genes of the third series and the relative abundance of nucleotides at each position (the consensus “IUPAC string”). The similarity index of each sequence to the consensus was defined in a final search all 19 sequences (Matrix 3). Genes identified as having upstream ARE sequences are listed in alphabetical order: afsA (nucleotides 7362–7389; accession number AB011413); a putative adhC gene (nucleotides 1450–1475 of cosmid SCD95A in the Sanger Center S. coelicolor Genome Database); the bar locus (accession number AB001690), including barB (nucleotides 134–161) and barA (BARE1, nucleotides 1506–1478; and BARE2, nucleotides 1267–1294); ccaR (nucleotides 1678–1703; accession number AF073897); the far locus (accession number AB001683), including farA (nucleotides 1429–1456) and farX (nucleotides 272–246); the jadR locus (accession number U24659), including jadR2/jadR1 (nucleotides 1819–1843 and 2025–2050); orbB (nucleotides 4570–4597; accession number AL132824); papR1 (nucleotides 1009–1037; accession number A37840); sbcR (ARE1, nucleotides 70–94; ARE2, nucleotides 324–349; accession number ATY02672); the tyl locus (accession number AF145049), including tylQ (nucleotides 4570–4597), tylP (nucleotides 2185–2209), and tylS (nucleotides 5671–5693); and varM (nucleotides 1651–1676; accession number AB035547). In the case of papR1, the footprinted region extended 5 bases beyond the conserved ARE motif. The data were compiled to define the frequency (consensus index) of bases (IUPAC string) in each position.
A nucleotide sequence matrix compiled from seven experimentally verified AREs (30) was used to search the Streptomyces nucleotide sequences in the complete GenBank™/EBI Data Bank and the Sanger Center S. coelicolor Database (>90% complete). Nineteen ARE-like sequences were detected and used to determine a set of matrix similarity indices that represented the relative adherence of each sequence to the most probable motif and the relative frequency of nucleotides at each position (IUPAC string) (Fig. 7B). These putative ARE-regulated genes include GABRs (scbR and tylP), SARPs (ccaR and tylS), proteins involved in butyrolactone biosynthesis (afaA and farX), and other genes within antibiotic biosynthetic clusters (jadR1, jadR2, umsR, and tylQ).

Of the three putative ARE-binding sites identified in the S. coelicolor genome, two were experimentally verified by gel retardation assay (data not shown) using both SpbR (purified) and crude extracts of E. coli producing recombinant ScbR (M15/pMF10) (see “Experimental Procedures”) (data not shown). These were located immediately upstream of scbR or between the 5′-sequences of adhC, a putative alcohol dehydrogenase, and an unidentified ORF. A putative site upstream of a histidine kinase paralog was located only 3 kb downstream of scbR.

A Constructed spbR Mutant Has Pleiotropic Defects in Pristinamycin Biosynthesis, Growth, and Aerial Mycelium Formation—To study spbR function, the gene was inactivated by insertion of an apramycin resistance cassette (aac(3)IV) into its unique MluI site (Fig. 2A). Diverse spbR-determined phenotypes were observed by comparing wild-type cultures with this mutant (spbR25) in various liquid and solid media. All phenotypes described below were suppressed by a plasmid containing spbR (pNL5) (Fig. 2A) and were therefore attributed to inactivation of spbR rather than polar effects on transcription of downstream genes or mutations in other loci. SpbR DNA-binding activity, assayed by gel retardation of the papRI fragment, was not detected in extracts from this mutant.

spbR25 grown in HT7T liquid medium did not produce any antibiotic activity detected with disc assays or the major secondary metabolite HPLC peaks characteristic of the wild-type strain. These included PI and PII as well as all of the other unidentified compounds (Fig. 6B), most of which belong to the pristinamycin complex. In addition, an unidentified dark pigment produced by the wild-type strain was not produced by spbR25.

Although spbR25 grew like its parent in HT7T liquid medium, colony growth and morphological development were very slow on corresponding agar-based solid media. Closer microscopic examination (Fig. 8) showed that germination and the earliest phase of colony development (the first 24 h) were...
similar in the wild-type and spbR25 strains. However, as the colony became barely visible, the mutant did not maintain wild-type rates of growth. Although the wild-type strain matured into much larger sporulating colonies, the growth and morphological development of spbR25 colonies were severely retarded. The mutant strain had similar effects on solid media tested (mannitol-soya, minimal production medium, NE, HTTT, and R2YE).

SbpR Is Needed for Expression of an Extracellular Superoxide Dismutase—SDS-polyacrylamide gel electrophoresis analyses (Fig. 9) showed the progressive accumulation of a major 23-kDa protein in NRR2958 mycelia growing in liquid cultures. This band was not detected in spbR25 unless the wild-type spbR gene was supplied in trans (pNL5) (data not shown). The N-terminal sequence of the 23-kDa protein eluted from either SDS-polyacrylamide or two-dimensional gels was the same: GTYALPDLPYDYSALAPAITPEILE. The sequence was identical to that of S. coelicolor A3(2) superoxide dismutase SodF at 19 (underlined) of 25 positions, suggesting that it is the N-terminal sequence of S. pristinaespiralis superoxide dismutase SodF.

This protein was independently identified as SodF by Western blotting using an antibody raised against M. tuberculosis SodF (Fig. 10A). The antibody detected a 23-kDa protein in cell extracts of NRR2958 that was not present in spbR25. As previously reported for S. coelicolor (16), expression of sodF in S. pristinaespiralis was suppressed by the addition of Ni^{2+} to the medium, but was not affected by chelation of divalent cations (Fig. 10A).

In situ detection of superoxide dismutase enzymatic activities resolved on native acrylamide gels confirmed these results. In the absence of supplemented NiCl_{2}, a single weak superoxide dismutase activity band, present in NRR2958 cultures, was missing in the mutant. NiCl_{2} suppressed accumulation of this protein, presumed to be SodF, and induced a slower migrating superoxide dismutase isoenzyme in both strains (Fig. 10B), presumed to be SodN.

Finally, SbpR shared an unusual feature with M. tuberculosis SodF. Western blotting using anti-M. tuberculosis SodF antibody detected an spbR-dependent 23-kDa protein in the medium (data not shown). SDS-polyacrylamide gel electrophoresis analysis of the total protein composition of the medium revealed that it was the only major band detectable by Coomassie Blue staining (Fig. 9, lane 8). Its N-terminal sequence (the first 5 residues were determined by Edman degradation) was identical to cytoplasmic S. pristinaespiralis SodF. Thus, both M. tuberculosis and S. pristinaespiralis accumulated SodF in the medium without the apparent N-terminal processing that characterizes type II protein secretion systems.

**DISCUSSION**

Our studies of S. pristinaespiralis SbpR extended several unifying concepts and established the principal functions of Streptomyces quorum-sensing signals and their receptors. Data base searches for corresponding operator sites revealed that, in addition to autoregulating their own expression and that of other genes involved in butyrolactone synthesis, GABR proteins may control SARPs, the primary class of antibiotic regulatory proteins in Streptomyces. However, other targets may play a different role related to pleiotropic growth defects outlined below.

Reinforcing previous reports, we concluded that SbpR-related GABRs play alternative physiological roles involving species-specific regulatory systems. In S. griseus, disruption of the A-Factor receptor leads to early differentiation and increased streptomycin biosynthesis. Inactivation of the barA gene in S. virginiae leads to precocious virginiamycin biosynthesis, but does not affect morphological differentiation (11). Although these data suggest inhibitory functions for butyrolactone receptors in other Streptomyces species, our results revealed positive roles for spbR in S. pristinaespiralis in maintaining growth, regulating antibiotic biosynthesis, and allowing a normal response to oxidative stress.

**Pleiotropic Effects of the spbR Mutation on Growth and Antibiotic Biosynthesis—spbR25 had a developmental growth defect on solid media. Just as the mycelial mass became barely visible on solid media, the mutant failed to maintain growth rates comparable to those of its non-defective parent. This may be interpreted as an inability to carry out a developmentally controlled metabolic transition allowing continued growth. Thus, the SbpR quorum sensor protein may facilitate recovery from a growth arrest that occurs during differentiation of several Streptomyces species (31–33).

Curiously, although growth of spbR25 cultures were not impaired in liquid cultures with the same composition as the HTTT solid medium, pristinamycin biosynthesis was still blocked. This probably resulted from distinct developmental programs reflected most obviously in morphological differences between mycelia grown on liquid versus solid media. For example, free circulation of the medium and fragmentation of mycelia in liquid cultures may allow better nutrient exchange or prevent localized accumulation of negatively acting waste products. These results suggest that the inability of spbR25 to produce pristinamycin is not a simple function of its conditional vegetative growth defect, an interpretation further supported by the fact that SbpR bound upstream of an SARP gene located within the pristinamycin biosynthetic cluster.

**Regulatory Targets of spbR—**This first demonstration that an autoregulator receptor protein interacted with an SARP gene promoter region led to the conclusion that AREs are primarily found upstream of genes involved in butyrolactone or antibiotic biosynthesis. DNase I footprinting showed that SbpR protected a sequence similar to those protected by BarA, and FarA (AREs) and provided the first experimental evidence that certain GABR proteins recognize heterospecific ARE motifs.
Only three AREs were clearly detected by the sequence matrix search of the *S. coelicolor* Database, which included at least 90% of the 8.7-megabase pair(s) chromosome. In addition to *sodR*, two uncharacterized genes of unknown function (one between *adhC* and an unidentified ORF (SCD95A) and the other upstream of a putative histidine kinase) were detected. This suggested that autoregulator receptor proteins do not interact directly with known SARP controlling undecylenic acid biosynthesis. However, the data cannot absolutely rule out the possibility that the matrix specifically identified only a subset of the ARE sequences under the control of GABR proteins in vivo.

Although it is not known whether *sodR* is genetically linked to *papR1*, these genes have several interesting similarities to the *S. fradiae* tylosin biosynthetic cluster (34). In both systems, AREs were identified upstream of SARP genes (*papR1* and *tylS*) and autoregulatory proteins (*sodR* and *tylP*). Furthermore, there was a syntenous arrangement of cytochrome P-450, the autoregulatory receptor (SodR or TylP), and acyl-CoA oxidase genes. These observations indicate that the two antibiotic regulatory systems utilize a conserved mechanism to coordinate host metabolism with antibiotic biosynthesis.

*SodR Is Needed for SodF Expression*—N-terminal sequence, *in situ* activity staining, and immunoblot analyses showed that *sodR* lack a major cytoplasmic protein identified as SodF. The *S. pristinaespiralis* 23-kDa superoxide dismutase was maintained at rather constant levels during growth and was repressed by NiCl₂, as previously reported for *sodF* in *S. coelicolor* and *Streptomyces lividans* (16). This, along with its size, cross-reactivity with antibodies raised against *M. tuberculosis* SodF, and N-terminal sequence, showed that it corresponds to the *S. pristinaespiralis* sodF gene.

SodF was also the major extracellular protein that accumulated in the medium of *S. pristinaespiralis* NRRL2958 cultures. The fact that both internal and external proteins had the same size and N-terminal sequences indicated that superoxide dismutase might well be autotransported as a leaderless protein (35). The extracellular SodF of *M. tuberculosis* may protect the pathogen from macrophage oxidative attack. Our observation of the same phenotype in a related saprophytic bacterium suggests a more generic metabolic function.

The extracellular SodF of *S. coelicolor* is exported in the medium of *S. coelicolor* controlled catalase studied in *Caulobacter crescentus*. Interestingly, in *C. crescentus* or *Streptomyces* (38). CatB, a developmentally controlled catalase studied in *S. coelicolor*, is required for aerial mycelium formation (38), which may depend on activation of oxidative metabolism (33, 39). Thus, an increased requirement for adaptation to oxidative stress, generated by metabolic shifts, may be an integral part of *Streptomyces* colonial development.

**Acknowledgment**—We are grateful to M. Horowitz for providing antibodies against *M. tuberculosis* SodF.

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Pleiotropic Functions of a Streptomyces pristinaespiralis Autoregulator Receptor in Development, Antibiotic Biosynthesis, and Expression of a Superoxide Dismutase
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J. Biol. Chem. 2001, 276:44297-44306.
doi: 10.1074/jbc.M101109200 originally published online September 13, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M101109200

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