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Global Negative Regulation of *Streptomyces coelicolor* Antibiotic Synthesis Mediated by an *absA*-Encoded Putative Signal Transduction System

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Streptomycete antibiotic synthesis is coupled to morphological differentiation such that antibiotics are produced as a colony sporulates. *Streptomyces coelicolor* produces several structurally and genetically distinct antibiotics. The *S. coelicolor absA* locus was defined by four UV-induced mutations that globally blocked antibiotic biosynthesis without blocking morphological differentiation. We show that the *absA* locus encodes a putative eubacterial two-component sensor kinase-response regulator system. All four mutations lie within a single open reading frame, designated *absA1*, which is predicted to encode a sensor histidine kinase. A second gene downstream of *absA1*, *absA2*, is predicted to encode the cognate response regulator. In marked contrast to the antibiotic-deficient phenotype of the previously described *absA* mutants, the phenotype caused by disruption mutations in the *absA* locus is precocious hyperproduction of the antibiotics actinorhodin and undecylprodigiosin. Precocious hyperproduction of these antibiotics is correlated with premature expression of XylE activity in a transcriptional fusion to an actinorhodin biosynthetic gene. We propose that the *absA* locus encodes a signal transduction mechanism that negatively regulates synthesis of the multiple antibiotics produced by *S. coelicolor*.

The widespread use of chemotherapeutic agents has been one of the most significant advances in medicine in this century. The vast variety of compounds available for human health care is due in large part to the biosynthetic versatility of the streptomycetes. These bacteria have provided many thousands of structurally diverse low-molecular-weight chemicals that are currently being exploited in both medicine and agriculture. Many streptomycete secondary metabolites have found commercial applications. Some of these are antibacterial drugs, including streptomycin and tetracycline; antiparasitic drugs such as avermectin; fungicidal agents such as polyoxin; antitumor drugs such as adriamycin; and immunosuppressive drugs such as rapamycin. The biosynthetic pathways that produce these compounds have received considerable attention over the last few decades (45, 57).

Advances in understanding the regulation of antibiotic synthesis in streptomycetes have come from the study of antibiotic synthesis in the genetically well-characterized strain *Streptomyces coelicolor*. The four antibiotics produced by *S. coelicolor*, actinorhodin (Act), undecylprodigiosin (Red), calcium-dependent antibiotic (CDA), and methylenomycin (Mmy), are biosynthetically and genetically distinct, genes for the synthesis of each of the antibiotics being found in genetically unlinked clusters. All four biosynthetic loci have been genetically well characterized (30, 59, 60, 70), and three biosynthetic gene clusters have been cloned: the *act* genes (43), the *red* genes (22, 44), and the *mmy* genes (14). Expression of *act* biosynthetic genes has been shown to depend on a gene, linked to the biosynthetic genes, designated *actII-ORF4* (23, 25, 53). Similarly, *redD*, which is linked to *red* biosynthetic genes, is required for expression of at least some *red* genes (49, 64). ActII-ORF4 and RedD have been termed pathway-specific activators, and numerous antibiotic biosynthetic gene clusters in a variety of streptomycetes require the function of a genetically linked pathway-specific activator for expression (11).

In addition to genes whose products specifically regulate the expression of one of the antibiotic gene clusters, a number of loci which contain genes that globally regulate more than one of the antibiotic biosynthetic clusters have been identified. Mutations in some of these, the *bld* loci, pleitropically block the synthesis of all four antibiotics as well as the production of sporulating aerial hyphae (10, 48, 54, 62, 69). Others genes, including *abaA* (24), *afsQ1-Q2* (33), and *afsR-K-R2* (32, 46, 63, 66), play a role in the regulation of two or three of the antibiotics.

Mutations in two loci, *absA* (3) and *absB* (2), are known to block the synthesis of all four antibiotics while having no pleiotropic effects upon morphological development. In addition, suppressers of *absA* mutations (*sab*) which restore antibiotic synthesis to normal or even greater than normal levels exist (56). We report here the cloning and characterization of the *absA* locus and present evidence that the *absA* locus encodes a signal transduction pathway which normally acts as a global negative regulator of antibiotic synthesis in *S. coelicolor*.

MATERIALS AND METHODS

Growth conditions. R2YE (29) was used for growth on plates unless otherwise indicated. Thiostrepton was added to a final concentration of 10 μg/ml as indicated below. Hygromycin was added to a final concentration of 100 μg/ml as indicated below. YEME medium (29) was used for liquid growth. Incubation conditions for cells growing on solid substrates or in liquid culture were as described elsewhere (29). Spores were harvested and stored as previously described (29). *Escherichia coli* was grown on L agar or in L broth (61).

Plasmids used and DNA manipulations. The isolation and manipulation of *Streptomyces* plasmid or chromosomal DNA were performed according to the procedures described by Hopwood et al. (29). Minipreparation DNA from the C542 transformants was prepared from overnight cultures grown on cellulose

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TABLE 1. Bacterial strains used in this study

Strain	Genotype	Reference
S. coelicolor	A3(2)	
J1501	*hisA1 uraA1 strA1*[a,b]	15
C505	*absA505 hisA1 uraA1 strA1*[a,b]	3
C542	*absA542 hisA1 uraA1 strA1*[a,b]	3
C554	*absA554 hisA1 uraA1 strA1*[a,b]	3
C577	*absA577 hisA1 uraA1 strA1*[a,b]	3
M138	*argA1 proA1 cysD18* SCP1	29
M145	Prototroph[a]	29
M124	*cysD18 proA1 argA1*[a]	29
C410	*absA542*::RS100 *hisA1 uraA1 strA1*[a,b]	This work
C411	*absA542*::RS100 *hisA1 uraA1 strA1*[a,b]	This work
C412	*absA*::RS110 *hisA1 uraA1 strA1*[a,b]	This work
C413	*absA*::RS120 *hisA1 uraA1 strA1*[a,b]	This work
C414	*absA*::RS120 *hisA1 uraA1 strA1*[a,b]	This work
C415	*absA*::RS130 *hisA1 uraA1 strA1*[a,b]	This work
C416	*absA*::RS140 *hisA1 uraA1 strA1*[a,b]	This work
C417	*absA505*::RS300 *hisA1 uraA1 strA1*[a,b]	This work
C418	*absA505*::RS310 *hisA1 uraA1 strA1*[a,b]	This work
C419	*absA*::RS120[a]	This work
C420	Δ*absA1*::*ermE hisA1 uraA1 strA1*[a,b]	This work
C421	Δ*absA1*::*ermE*[a]	This work
C430	Δ*absA2*::RS500 *hisA1 uraA1 strA1*[a,b]	This work
1501/KC900	*actI*::KC900 *hisA1 uraA1 strA1*[a,b]	9
C425	Δ*absA1*::*ermE actI*::KC900 *hisA1 uraA1 strA1*[a,b]	This work
S. lividans 1326		29
E. coli ET12567		41

[a] SCP1$^-$ SCP2$^-$ (two indigenous plasmids of *S. coelicolor*; SCP1 contains the biosynthetic loci for the antibiotic methylenomycin [70]).
[b] Pgl$^-$ (phage restriction system) (38).

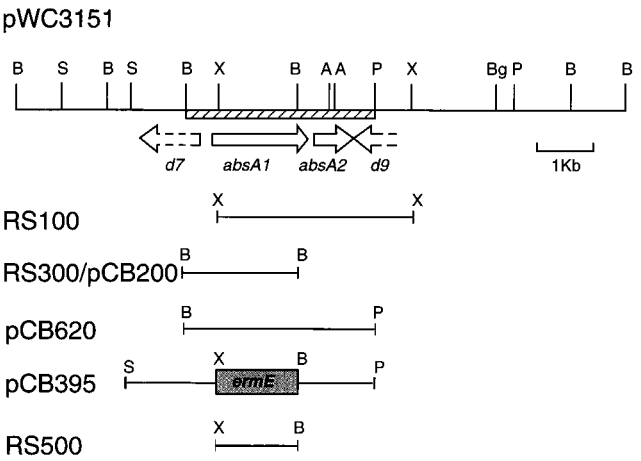

FIG. 1. Restriction map of the insert in pWC3151. (Top) A restriction map of the insert in pWC3151 is shown along with the positions of the four ORFs identified in this study. (Bottom) RS100 was created by cloning the 3.2-kb *Xho*I fragment into KC516 (58). The 2-kb *Bam*HI fragment was introduced into KC516 to generate RS300 and into the low-copy-number, self-mobilizing plasmid pIJ922 (40) to generate pCB200. pCB620 was created by subcloning the 3.2-kb *Bam*HI-*Pst*I fragment into pIJ922 (via the polylinker of pIJ2925 [34]). pCB395 was created by subcloning the 3.8-kb *Sac*I-*Pst*I fragment into pIJ2925 and then replacing the 1.45-kb *Xho*I-*Bam*HI fragment with a cassette containing the *ermE* gene. The 1.45-kb *Xho*I-*Bam*HI fragment, which is internal to *absA1*, was ligated into the *Xho*I-*Bgl*II sites of KC516 to create RS500. Restriction sites: A, *Apa*I; B, *Bam*HI; Bg, *Bgl*II; P, *Pst*I; S, *Sac*I; X, *Xho*I.

acetate discs on L agar. The mycelium was gently removed from the surface of the plates before the DNA was isolated by the procedures described by Hopwood et al. (29).

Construction of a pIJ922 library and conjugal transfer. The library was prepared by size fractionating partially *Sau*3A-digested J1501 chromosomal DNA on a sucrose gradient. DNA fragments in the 10- to 30-kb size range were isolated and ligated into the unique, CIAP-dephosphorylated *Bam*HI site of the SCP2*-based vector pIJ922. The library was used in a polyethylene glycol-assisted transformation of strain M124 (29). Approximately 3×10^4 transformants were selected and patched onto R2YE plates. Following sporulation, colonies were replicated onto lawns of C542 spores which had been seeded onto R5 plates. After sporulation, these plates were replicated onto a minimal medium which selected for exconjugants (i.e., against the M124 genotype [*cysD18 proA1 argA1*] but for the C542 genotype [*hisA1 uraA1 strA1*] and the thiostrepton resistance associated with the plasmid).

Antibiotic assays. Assays for the detection of actinorhodin, undecylprodigiosin, CDA, and methylenomycin were performed as described by Adamidis et al. (3). For methylenomycin assays, a C542-derivative carrying the methylenomycin-encoding plasmid SCP1 was used; this strain was constructed by crossing SCP1 from strain M138 (Table 1). *absA*$^+$ clones (e.g., pWC3151) were then crossed into C542 SCP1$^+$, with selection for Thio^r. For comparisons of actinorhodin and undecylprodigiosin production for C420 and J1501, the strains were grown on cellophane discs for 75 h, at which time the mycelium was harvested and suspended in 6 ml of water, with 3 ml being used for each antibiotic assay.

Construction of lysogens in *S. coelicolor* and the harvesting of excised prophage for marker exchange experiments. Actinophage suspensions were prepared according to the protocols of Hopwood et al. (29). Spores of the host strain were plated onto R5 medium, and an aliquot of the phage suspension was spotted onto the lawn of spores. Following sporulation, the plates were replicated onto selective minimal medium that contained thiostrepton at 20 μg ml^{-1}. The phenotypes of thiostrepton-resistant colonies were characterized. The genotype of lysogens was verified by Southern blot analysis of digested chromosomal DNA, which was hybridized to various probes from the *absA* locus. Excised phage were harvested by replicating plates of the lysogen onto nutrient agar plates overlaid with *Streptomyces lividans*-seeded soft nutrient agar, soaking out phage into Difco nutrient broth, and then filtering the suspension through a 0.2-μm-pore-size filter. The phage suspension was then screened for phage with the correct genotype.

DNA sequencing. For DNA sequencing the dideoxy-chain termination method was used (Lark Sequencing Technologies Inc.). 7-Deaza-dGTP or 7-deaza-dITP was used, if necessary, to resolve severe GC band compressions. DNA templates were generated by exonuclease III digestion (27) and cloned into Bluescript vectors. Every part of the sequence was determined at least twice in both directions. For sequence analysis the Wisconsin Genetics Computer Group package (20) was used.

Construction of the *absA1* null mutants C420 and C421. The 3.7-kb *Sac*I-*Pst*I fragment was subcloned from pWC3151 into the Bluescript vector pSK$^+$ and from there into pIJ2925. The 1.4-kb *Xho*I-*Bam*HI fragment, which encodes amino acids 47 through 502 of the *absA1* gene product, was replaced by the *ermE* gene (7), inserted such that the transcription of *ermE* was opposite to that of the *absA* locus. This construct was released as a *Bgl*II fragment and cloned into the *Bam*HI site of pIJ963, creating pCB395 (Fig. 1). This plasmid was passaged through the methylase-deficient *E. coli* strain ET12567 (Table 1). The DNA extracted from this strain was used to transform the protoplasts of two strains: J1501 (the auxotrophic parent strain for the original *absA* mutants) (3) and M145 (a prototrophic wild-type strain). Transformants were selected for resistance to both erythromycin and hygromycin; this would select for single crossovers. Spores were harvested from representative transformants and plated onto nonselective medium. Once these colonies had sporulated, they were replicated onto medium containing either erythromycin or hygromycin. From this screen Hyg^s Ery^r colonies were isolated. Chromosomal DNA, digested with *Kpn*I and *Xho*I and probed with flanking regions of the *absA1* gene, contained the fragments predicted for the pattern of an *ermE* disruption of *absA1* (data not shown). Representative Δ*absA1*::*ermE* derivatives of J1501 and M145 were named C420 and C421, respectively (Table 1).

XylE enzyme assays. The growth conditions and assay techniques for the KC900 and KC902 lysogens were the same as for XylE assays described previously (65), except the spores were plated on SPMR medium (4). The KC900 phage contains a fragment internal to the *actI* coding region and creates lysogens through homologous recombination with *actI*. Thus, it creates a single-copy transcriptional fusion of the *actI* promoter to the reporter gene *xylE* (9). Spores were inoculated onto cellophane disks on plates of SPMR medium, the plates were incubated for various lengths of time, cultures were harvested, and each sample was assayed twice for XylE activity.

Nucleotide sequence accession number. The DNA sequence reported here has been deposited in the GenBank database under accession number U51332.

RESULTS

Cloning a sequence that restores antibiotic production to *absA* mutants. The original *absA* mutants were isolated by taking advantage of the fact that two of the antibiotics are pigments (59, 60). The pigment-deficient phenotype of the *absA* mutants is dramatically distinct from that of the deeply

TABLE 2. Plasmids and bacteriophages used in this study

Plasmid or phage	Description	Reference
Streptomyces plasmids		
pIJ922	SCP2*-based low-copy-number plasmid	40
pWC3151	pIJ922 carrying 11.5-kb complementing insert	This work
pCB620	pIJ922 carrying 3.2-kb *Bam*HI-*Pst*I insert	This work
pCB200	pIJ922 carrying 1.95-kb *Bam*HI insert	
E. coli plasmids		
pSK^+/pKS^+ Bluescript		Stratagene
pIJ2925	pUC19 derivative with modified polylinker	34
pIJ963	pIJ2925 containing *hyg*	36
pIJ395	pIJ963 containing *Sac*I-*Pst*I insert	This work
Streptomyces phages		
KC516	ϕC31-derived actinophage, $att^- c^+$	58
KC900	*actI*::*xylE* transcriptional fusion, $att^- c^+$	9
KC902	*redX*::*xylE* transcriptional fusion, $att^- c^+$	26
RS100	KC516 carrying 3.2-kb *Xho*I insert	This work
RS300	KC516 carrying 1.95-kb *Bam*HI insert	This work
RS500	KC516 carrying 1.45-kb *Xho*I-*Bam*HI insert	This work

pigmented parental strain J1501, and complementing clones should restore the color. However, initial attempts to isolate complementing DNA were complicated by two phenomena. First, suppressive *sab* mutations, which created an Abs^+ phenotype (56), arose spontaneously in the *absA* mutant background at a frequency such that *sab* pseudorevertants were reproducibly present at a frequency of approximately 0.1% in *absA* protoplast preparations. Second, *absA* mutant protoplasts transformed at a frequency approximately 25-fold lower than either the wild-type strain J1501 or the *sab* pseudorevertant strains. The impact of these two factors was that the background level of phenotypically Abs^+ transformants arising after introduction of a cloning vector containing no insert was as high as 2.5%.

To circumvent these problems, we employed a cloning scheme based on the introduction of a library by conjugal transfer of the self-mobilizing plasmid pIJ922 (19) (see Materials and Methods). After mating into the *absA* strain C542 (Table 1), clones which gave Abs^+ or pigmented patches, with approximately wild-type levels of pigment, were isolated. When used to transform protoplasts of C542, eight independent clones, which had been identified by the mating procedure, reproducibly conferred the Abs^+ pigmented phenotype. The putative $absA^+$ clones were also introduced into other *absA* mutants, C505, C554, and C577 (Table 1), resulting in production of actinorhodin and undecylprodigiosin.

When tested for the ability to restore biosynthetic capacity of CDA (see Materials and Methods), the putative *absA* clones were all found to restore antibiotic biosynthesis to wild-type levels (data not shown). Two representative clones were chosen for the methylenomycin assay (see Materials and Methods) and were shown to restore biosynthetic capacity for this antibiotic as well (data not shown). Thus, the putative *absA* clones globally restored antibiotic synthesis to *absA* mutant strains.

Preliminary restriction mapping of the putative *absA* clones revealed that four of the clones contained a comigrating 3.2-kb *Xho*I fragment, suggesting that all came from the same region. A representative clone, pWC3151 (Table 2), was chosen for further study. Restriction mapping of the cloned insert revealed that the 3.2-kb *Xho*I fragment lay in the center of the approximately 11.5-kb insert sequence (Fig. 1). Southern blot hybridizations revealed that at least a portion of this 3.2-kb *Xho*I fragment was shared by six of the remaining seven clones. A seventh clone, pWC3146, showed no hybridization under stringent conditions, as will be reported elsewhere.

Marker rescue of *absA* mutations in the chromosome. If the 3.2-kb *Xho*I fragment contained at least part of the *absA* locus, then recombination between the *Xho*I fragment and a mutant *absA* gene in the chromosome might give rise to some $absA^+$ recombinants due to marker rescue. To test for marker rescue the *Xho*I fragment was cloned into the Δ*attP* temperate actinophage KC516 (58), creating RS100 (Fig. 1). Because it lacks an *attP* region, lysogens resulting from infection by RS100 should be due to homologous recombination between the *Xho*I fragment and the corresponding region in the chromosome. If the clone overlaps the region of the *absA* mutation, some of these recombinants should be Abs^+. Approximately 50% of the lysogens produced pigment; the other lysogens retained the Abs^- phenotype. Similar results were obtained when RS100 was used to create lysogens in strains containing the other *absA* mutations. A straightforward interpretation of these data was that the 3.2-kb *Xho*I fragment spanned the region on the chromosome that contained the *absA* mutations but contained only one end of the sequence encoding the function altered by *absA* mutations (Fig. 2A). To identify fragments containing the entire *absA* encoding sequence, fragments overlapping the 3.2-kb *Xho*I fragment were also cloned into the phage. A 1.95-kb *Bam*HI fragment that shares a 1.45-kb overlap with the *Xho*I fragment (Fig. 1) was able to restore the Abs^+ phenotype to 100% of lysogens in each of the four strains carrying an *absA* mutation (Fig. 3). In addition, a plasmid containing the 1.95-kb *Bam*HI fragment, pCB200 (Fig. 1), restored the Abs^+ phenotype to all four *absA* mutants. These results suggested that the 1.95-kb *Bam*HI fragment spanned the sites of the *absA* mutations on the chromosome and included sufficient coding information to produce the functional protein.

Marker exchange demonstrated that the complementing sequence includes the $absA^+$ allele. The possibility that the clones contained a sequence with suppressive capacity and not the bona fide *absA* locus remained. This possibility could be eliminated if *absA* mutations could be crossed onto the clone. To determine if any of the phage released from the lysogens now had the *absA* mutant allele instead of the wild-type allele, the phage were harvested (Fig. 2B) and used to lysogenize the Abs^+ J1501 strain; approximately 50% of the lysogens were Abs^- (Fig. 2C). In contrast, stocks of RS100 intro-

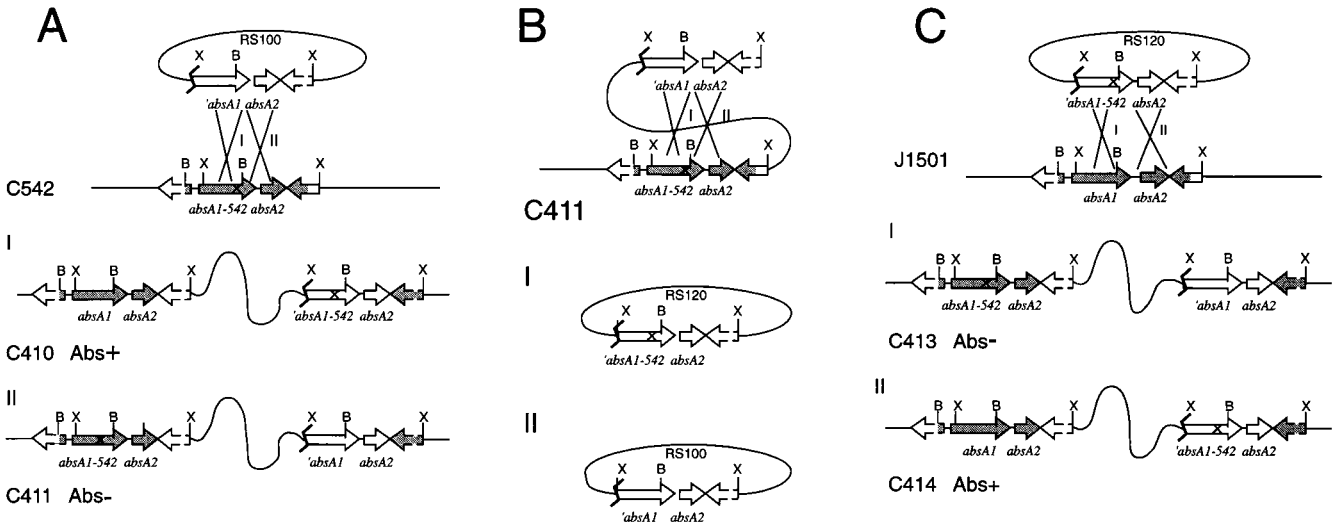

FIG. 2. The *Xho*I fragment in RS100 spans all four *absA* mutations. The shading indicates those ORFs likely to be expressed on the basis of sequence analysis in Fig. 4. ×, mutant site. (A) Recombination at position I would result in an Abs^+ (C410) phenotype, while recombination at position II would result in an Abs^- (C411) phenotype. (B) As the RS100 fragment spanned the *absA* mutations, some of the prophage released from the lysogens had recombined at position I and therefore picked up the *absA1-542* mutation on the phage genome, generating RS120. (C) Any phage able to create Abs^- lysogens would have to be carrying the *absA1-542* mutation and recombine at position I.

duced into J1501 produced no Abs^- lysogens of several hundred tested.

An additional experiment indicated that all four *absA* mutations lay within the 1.95-kb *Bam*HI region. To demonstrate this, phage were harvested from lysogen C417 and screened for phage that carried the *absA505* allele (see Materials and Methods). These phage could not be screened for in J1501, as all lysogens produced from either RS300 or RS310 (*absA505*) would be Abs^+. However, the phage were distinguished when introduced into the *absA* mutant C505, where RS300 produced

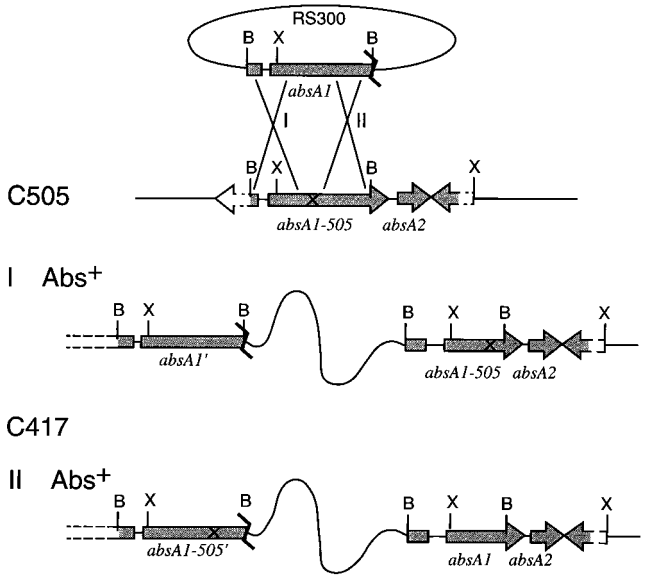

FIG. 3. The 2-kb *Bam*HI fragment spans the *absA* mutations, thus placing all four of the mutations in *absA1*. The shading is as discussed in the Fig. 2 legend. Marker exchange experiments were performed (Fig. 2B and C) with phage released from C417, and the *absA1-505* mutant allele was cloned in phage RS310.

Abs^+ lysogens and RS310 produced Abs^- lysogens, a result that could occur only if the RS300 insert spanned the *absA505* mutant site.

Correspondence of the genetic and physical map locations of the *absA* locus. Additional proof that the cloned sequence encoded the *absA* locus came from mapping the cloned DNA on the *S. coelicolor* chromosome. A combined physical-genetic *Ase*I chromosomal map has recently been constructed for *S. coelicolor* (strain M145) by pulsed-field gel electrophoresis (35). This allowed us to locate the 3.2-kb *Xho*I fragment of RS100 on the chromosome. A ^{32}P-labeled probe hybridized to *Ase*I fragment E (data not shown). This physical location of the *Xho*I fragment on the chromosomal map, at fragment E, correlated with the previously defined map position of the *absA* locus in the 10 o'clock region of the genetic map (3).

Nucleotide sequence and identification of the *absA1* and *absA2* genes. The 1.95-kb *Bam*HI fragment was sequenced. Computer-aided analysis (see Materials and Methods) of the sequence predicted two divergent open reading frames (ORFs) (Fig. 4). The smaller partial ORF, designated *d7*, was predicted to start at the ATG codon at nucleotides 310 to 312, with the 3′ end of the gene being downstream of the *Bam*HI site. The larger, divergent, ORF was predicted to start at the ATG start codon at nucleotides 447 to 449. This second ORF spanned the 1.45-kb *Xho*I-*Bam*HI region that contained the *absA* mutations. This gene was designated *absA1*. Because there were no stop codons in this reading frame before the *Bam*HI site, the sequence of the downstream 1.2-kb *Bam*HI-*Pst*I fragment was determined and a stop codon was found at nucleotides 2160 to 2162 (Fig. 4). A third ORF, downstream of *absA1* and transcribed from the same strand as *absA1*, started at the ATG codon at nucleotides 2181 to 2183. The 3′ end of this gene was defined by the stop codon at nucleotides 2847 to 2849. This ORF was designated *absA2* because of the relationship of its predicted product and that of the *absA1* gene (see below). The stop codon for *absA2* is over lapped by a second stop codon, nucleotides 2846 to 2848, for a predicted convergent ORF, designated *d9*, which is truncated at nucleotide 3216.

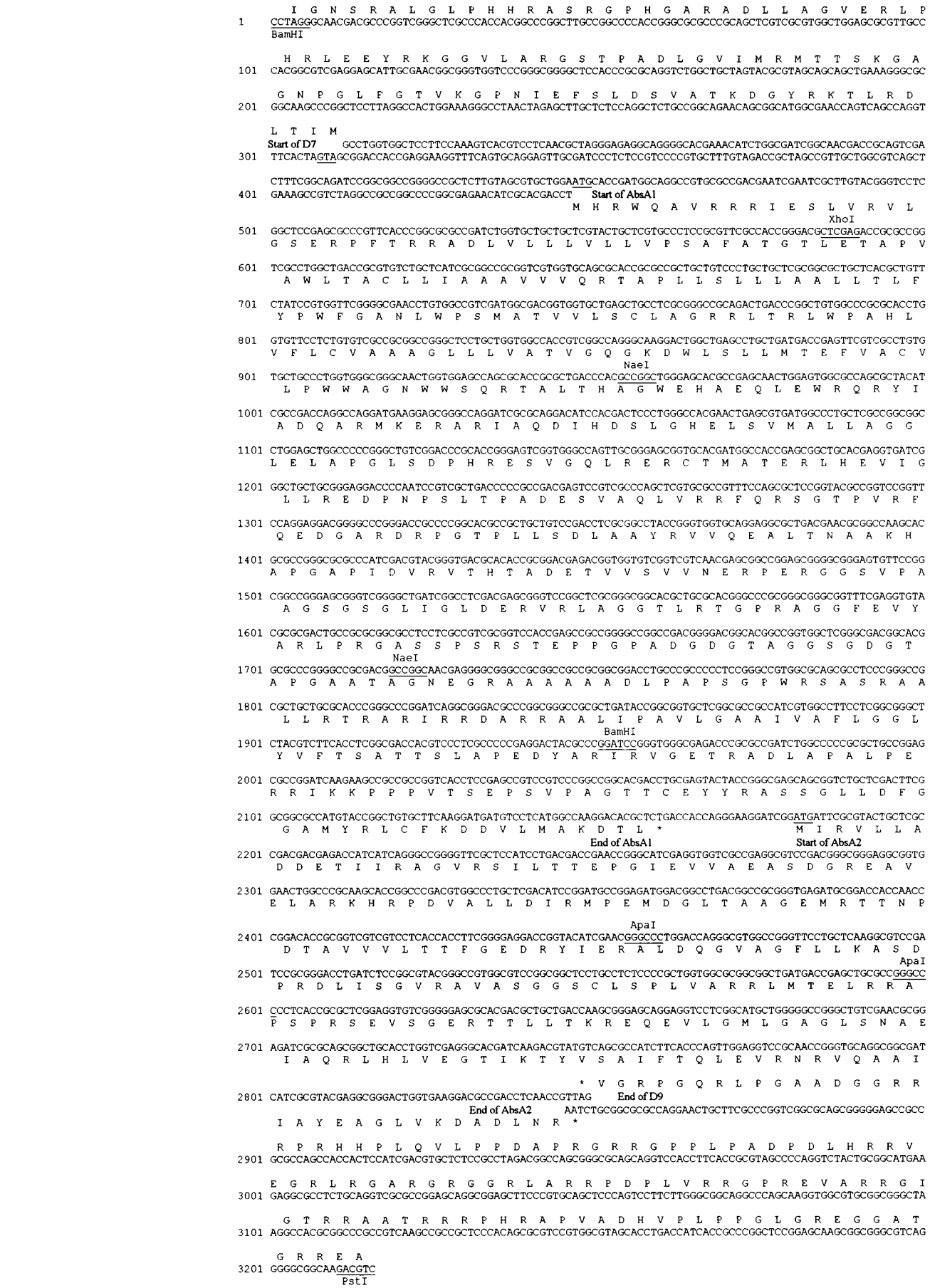

FIG. 4. Nucleotide sequence of the *absA* locus and the predicted amino acid sequence for the gene products of *absA1-A2*, *d7*, and *d9* are shown below the nucleotide sequence.

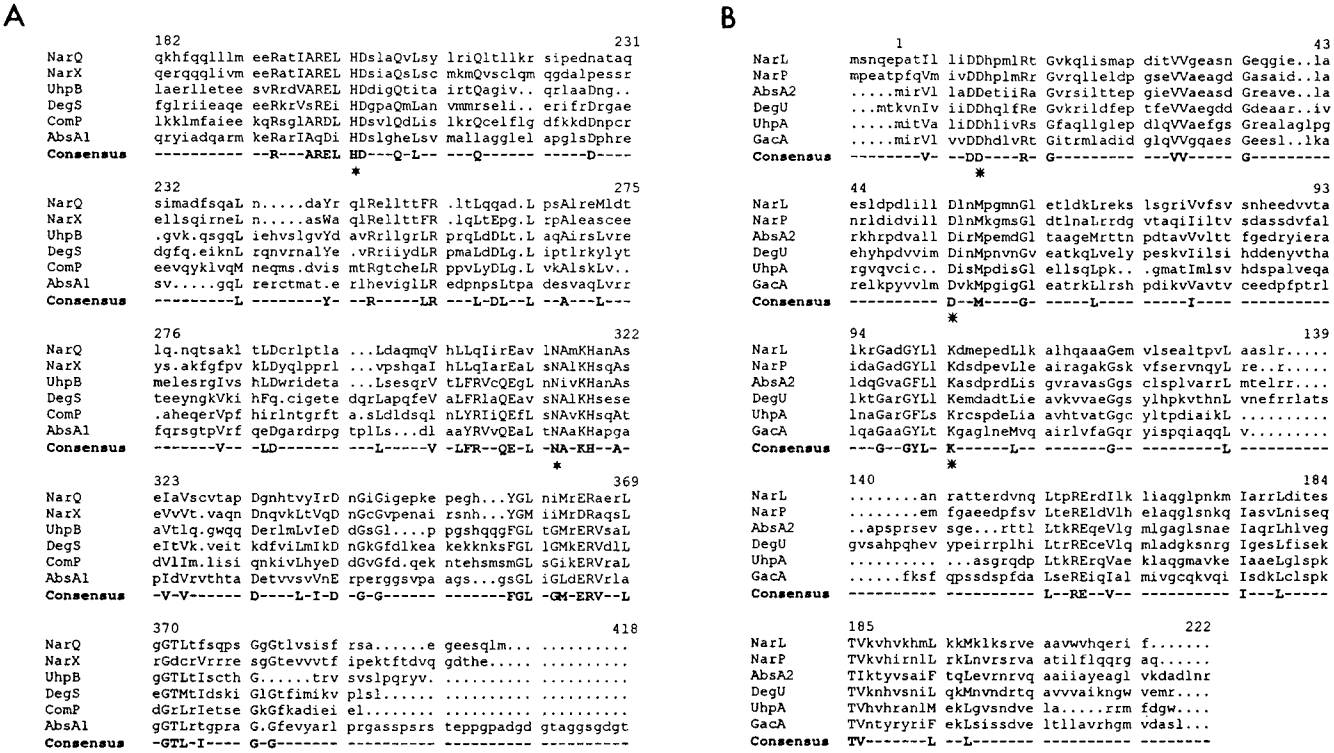

FIG. 5. Amino acid alignments for AbsA1 and AbsA2 with eubacterial two-component systems from the Deg/Uhp subgroups. (A) Sequence similarities between AbsA1 and representative members of the DegS/UhpB subgroup of histidine kinases: NarQ from *E. coli* (16), NarX from *E. coli* (50), UhpB from *E. coli* (68), DegS from *B. subtilis* (28), and ComP from *B. subtilis* (67). Conserved residues are recorded in boldface in the consensus sequence; the two highly conserved amino acids in histidine kinases are also indicated (asterisks). The numbers refer to the amino acids of the AbsA1 polypeptide. (B) The sequence similarities between AbsA2 and representatives of the DegU/UhpA subgroup of response regulators: UhpA from *E. coli* (68), NarL from *E. coli* (50), NarP from *E. coli* (55), GacA from *P. fluorescens* (39), and DegU from *B. subtilis* (28). Conserved residues are recorded in boldface in the consensus sequence; the three highly conserved amino acids in response regulators are also indicated (asterisks). The numbers refer to the amino acids of the AbsA2 polypeptide. Capital letters indicate conserved residues; dots indicate gaps introduced to maximize alignment; dashes in the consensus sequence indicate a lack of consensus.

The *absA* locus encodes a putative two-component signal transduction system. A computer-aided search of the GenBank and EMBL databases revealed that AbsA1 showed significant sequence similarities to eubacterial histidine kinases (52), particularly to the UhpB/DegS subgroup that also contains ComP, NarQ, and NarX (52) (Fig. 5A). The similarities among the histidine kinase superfamily of proteins occur in several conserved domains, the most notable of which is centered around the autophosphorylated histidine (52). It is predicted, from the sequence alignments, that the histidine at residue 202 is part of this conserved histidine phosphorylation domain in AbsA1 (52) (Fig. 5A). There is a second conserved domain centered around an asparagine residue (52) that aligns with the AbsA1 asparagine residue at position 314 (Fig. 5A). The COOH terminus of the AbsA1 protein shows little or no sequence similarity with the other histidine kinases and is longer than comparable regions in most histidine kinases. The COOH termini of the histidine kinases are variable, possibly because of the variety of functions performed by the various histidine kinases (52). The COOH-terminal 69 amino acids of AbsA1 were not required to complement the *absA* mutant alleles (Fig. 3). Like many histidine kinases, the amino terminus of AbsA1 contains several hydrophobic domains that may be involved in spanning the membrane of the cell (52).

The AbsA2 polypeptide showed significant sequence similarities to members of the UhpA/DegU subgroup of response regulator proteins that function with the UhpB/DegS group of histidine kinases (52) (Fig. 5B). For the response regulators, the regions with the highest sequence similarities are located in the amino-terminal and central portions of the proteins; these include two conserved aspartate residues, one of which is the site of phosphorylation, and a conserved lysine residue (52). Alignment of AbsA2 with the other response regulators leads us to predict that residue 54 (Asp) is the probable site of phosphorylation (Fig. 5B). The level of sequence identity is much higher among the response regulators than among histidine kinases (52), and AbsA2 shows significant levels of identity, 39% with DegU and 35% with NarP, across its entire length.

Mutational disruption of the *absA* locus resulted in precocious hyperproduction of antibiotics. The apparent rarity of the original AbsA mutant isolates had suggested that the mutant strains do not contain null alleles (3). To address this issue, a defined deletion was created in the *absA* locus. This was constructed by replacing amino acids 47 (Leu) to 502 (Arg) of the *absA1* gene with the resistance marker *ermE* (see Materials and Methods). This deletion-replacement construction deleted all conserved domains of AbsA1, leaving only 69 C-terminal amino acids and 46 N-terminal amino acids. This replacement construction would likely have a polar effect on expression of *absA2*. We have not yet determined whether *absA2* is obligatorily cotranscribed with *absA1*, but the close linkage of the ORFs (Fig. 4) and precedent of other two-component gene pairs (52) suggest that it is. Transcription of *ermE* was opposite to that of *absA1* and *absA2*, so read-through transcription would not transcribe *absA2*.

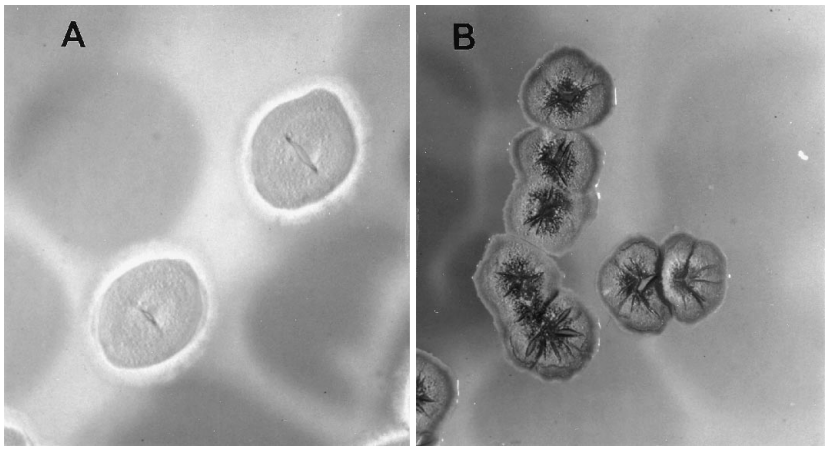

FIG. 6. (A) The J1501 colonies have a regular domed-shaped surface which is covered with aerial hyphae and mature, grey spore chains. (B) The isolated colonies of C420 have an irregular crenulated surface which is only sparsely covered with aerial hyphae and mature spore chains. The C420 spores were viable and appeared, in phase-contrast microscopy, indistinguishable from J1501 spores. The severity of the morphological phenotype was dependent upon the colony density on the agar plate, with the most densely populated areas showing colony morphology more reminiscent of the parent strain but the more isolated colonies (as shown) showing the pronounced crenulated phenotype. The C420 colonies were also routinely smaller in diameter than those of their parent, J1501.

The visible phenotype and measurements of actinorhodin and undecylprodigiosin synthesis in a representative *absA*::*ermE* mutant strain, designated C420 (Table 1), indicated that both actinorhodin and undecylprodigiosin were produced prematurely and much more abundantly in the *absA1* null mutant in comparison to the parent strain J1501. In three replicate assessments of plate-grown cultures, actinorhodin production initiated 6 to 12 h earlier in C420 and undecylprodigiosin production initiated 6 to 12 h earlier. At 75 h of incubation, the C420 culture produced fivefold-increased actinorhodin and eightfold-increased undecylprodigiosin with respect to amounts produced by J1501 (0.74 versus 0.14 mg of actinorhodin per g of mycelium and 0.83 versus 0.11 mg of undecylprodigiosin per g of mycelium). C420 produced abundant CDA, but the zone-of-killing assay used (see Materials and Methods) did not conclusively demonstrate an increased rate of production in C420. Methylenomycin production was not assessed.

The colony morphology of the C420 colonies was also altered: the surface of the colony was highly crenulated, and sporulating hyphae were sparse (Fig. 6). In comparison to J1501, these mutants also show a marked deficiency in their transformation efficiency, comparable to the transformation deficiency described for the C542 protoplasts. We do not have an explanation for the transformation deficiency, nor do we yet understand the relationship between the antibiotic production and morphological phenotypes of C420. To verify that all the phenotypes described for C420 were due solely to the disruption of the *absA* locus, plasmid pCB620 (Fig. 1) was introduced into this strain by transformation. Transformants were restored to the wild-type phenotypes for all characteristics described above.

Disruption of *absA2* alone may cause precocious overproduction of antibiotics. All aspects of the C420 phenotype were duplicated in a strain, C430, in which the *absA* locus was disrupted as shown in Fig. 7. To construct C430, phage RS500, containing an insert internal to *absA1* (Fig. 1), was introduced into J1501 by homologous recombination at the *absA1* gene. C430 would express a truncated version of AbsA1, but because the same truncated version of AbsA1 was sufficient for complementation of the *absA1-505* mutant (as shown in Fig. 3), it is possible that the C430 mutant phenotype resulted from a failure to express *absA2*.

Precocious production of actinorhodin correlated with premature expression of a *xylE* transcriptional fusion to an antibiotic biosynthetic gene. To assess whether the increase in actinorhodin production in C420 was due to an increase in the transcription of the *act* biosynthetic genes, a transcriptional fusion was constructed between an *act* biosynthetic promoter, *actI*, and the reporter gene *xylE* by using actinophage KC900 (9) (see Materials and Methods). The XylE activity of the C420::KC900 strain (C425) (Table 1) initiated between 48 and 54 h after inoculation on SPMR medium; this was consistently 6 to 12 h before the onset of XylE activity from the J1501::KC900 strain. Also, the peak activity for C420::KC900 occurred earlier and was greater than that for J1501::KC900 (Fig. 8). The onset of actinorhodin production was also assayed during the time course and showed a temporal difference for the onset of production corresponding to the difference seen in the XylE transcription assays (Fig. 8). Similar experiments were carried out using the less well characterized KC902 *xylE* fusion to the *redX* undecylprodigiosin biosynthetic locus (26). In these experiments, XylE activity in C420::KC902 increased

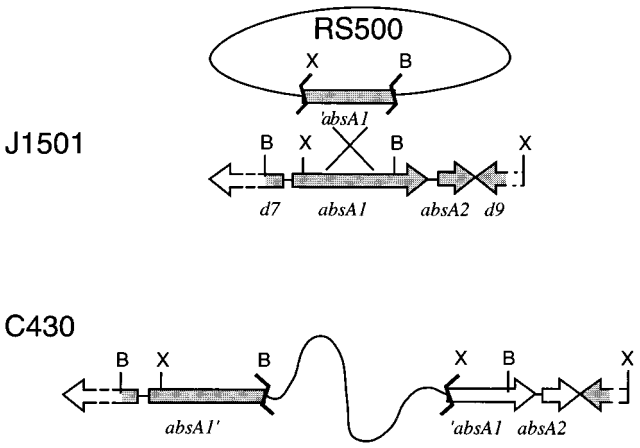

FIG. 7. Disruption of the *absA* locus by integration of RS500. The shaded areas represent those ORFs thought to be transcribed in these constructs.

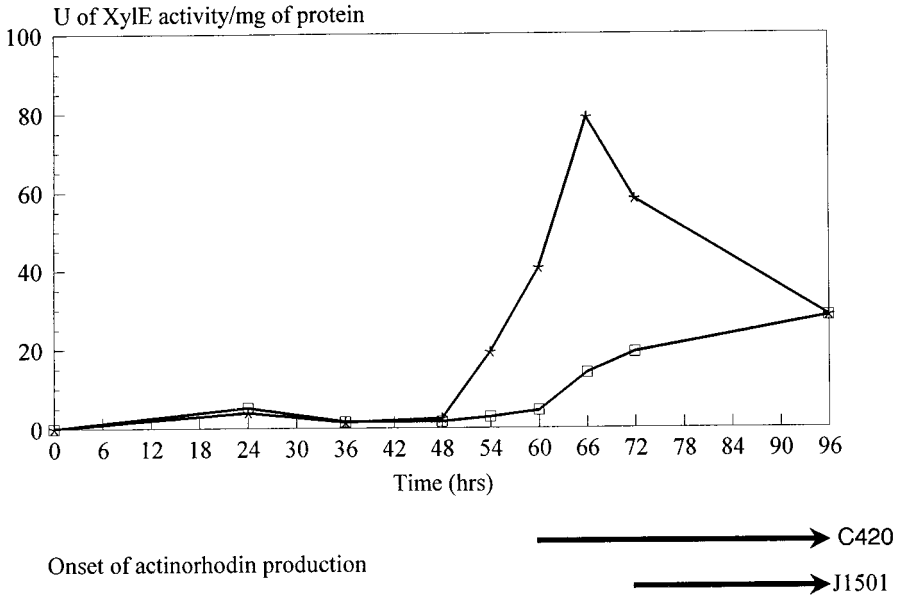

FIG. 8. Expression of an *actI*::*xylE* transcription fusion. This time course was repeated twice, and the temporal difference in *actI*::*xylE* expression between C420::KC900 (*) and J1501::KC900 (□) ranged between 6 to 12 h for both time courses. The arrows at the base of the figure illustrate when actinorhodin was first produced during the assays.

6 to 12 h earlier than in J1501::KC902. Peak XylE activity in C420::KC902 was approximately 20% higher than in J1501::KC902 (data not shown). Because *red* transcripts are not well characterized, transcription from *red* genes, in the C420 strain, will require further characterization.

The *absA1-542* and Δ*absA1*::*ermE* mutant alleles caused identical phenotypes in a range of laboratory *S. coelicolor* strains. Much of the molecular genetic investigation of *S. coelicolor* antibiotic regulation has utilized two strains as representatives of wild-type antibiotic production and regulation characteristics: J1501 (2, 3, 23) and M145 (24, 25, 64). Therefore, we constructed *absA542*-carrying strains with J1501 and M145; these were named C413 and C419, respectively. To create C419, the *absA542* allele was introduced into M145 on phage RS120, (similarly to the protocol of Fig. 2C). C419, like C413, failed to produce any detectable actinorhodin, undecylprodigiosin, or CDA (methylenomycin production was not assessed since M145 lacks the methylenomycin-encoding plasmid SCP1). Also, there were no obvious deficiencies in the sporulation or growth of C419.

By the same procedure as was used for the construction of C420 (see Materials and Methods) the *absA1*::*ermE* allele was introduced into M145, creating C421. The phenotype of C421 was precocious hyperexpression of antibiotics, as has already been described for C420. Similarly, C421 displayed the crenulated surface morphology and transformation deficiencies described for C420. Thus, the phenotypes seen in the M145 background are identical to those described for the *absA* alleles in a J1501 background.

DISCUSSION

We have shown that the *absA* locus exerts global control over production of the *S. coelicolor* antibiotics. The *absA* locus was first defined by mutations that globally blocked antibiotic synthesis. All four such mutations lie within *absA1*, which is predicted to encode a sensor kinase. Downstream of *absA1* lies a second ORF, *absA2*, which is predicted to encode the cognate response regulator for AbsA1. In stark contrast to the antibiotic-nonproducing phenotype resulting from the original *absA* mutations, disrupted *absA* alleles, as exemplified in the C420 and C430 strains, cause an opposite effect on antibiotic synthesis—precocious overproduction. Early, increased activity of XylE in *act*::*xylE* and *red*::*xylE* fusion strains correlates with the observable enhanced antibiotic synthesis in the C420 strain.

Fundamental studies of *S. coelicolor* physiology and genetics have utilized a number of laboratory strains. Our choice of strain J1501 as the parent for mutant isolation schemes was based on its suitability for subsequent genetic analyses; its attributes include useful markers for genetic mapping studies and suitability as a host for phage and plasmid vectors (29). Furthermore, in contrast to some other *S. coelicolor* strains that exhibit variability in morphological and antibiotic phenotypes—a widely observed characteristic of *Streptomyces* spp. that is associated with chromosomal rearrangements (8)—J1501 is phenotypically relatively stable. This attribute obviously facilitated mutant screens. But J1501 has recently been shown to have suffered deletion of as much as 1/10 of its genome (35). Clearly it was important for us to contrast the phenotypic effects of the mutant *absA* alleles in J1501 and other, undeleted strains. As we show above, both the *absA542* blockage to antibiotics and the Δ*absA1*::*ermE*-effected precocious synthesis of antibiotics occur when these alleles are transferred to another well-characterized *S. coelicolor* strain.

The premature hyperexpression of antibiotics in C420 and C430 may be due to absence of an AbsA2 response regulator. Thus, an *absA*-encoded two-component system may function as a negative regulator of antibiotic gene expression. Global negative regulators of *S. coelicolor* antibiotic biosynthesis other than *absA* have not been described, but it is not unprecedented for response regulators to function in negative as well as positive regulation. One such example is the DegS/U system from *Bacillus subtilis*, in which the response regulator DegU plays multiple roles during the transition from exponential growth to stationary phase (18). The phosphorylated form performs dual

functions: it represses *srfA*, which is involved in the production of the antibiotic surfactin, and it positively regulates degradative enzyme synthesis. The unphosphorylated form is also biologically active and plays a role in the acquisition of genetic competence. At present the exact mechanism of the AbsA-mediated negative regulation is not known. But as we have shown, AbsA signal transduction exerts its effect on transcription of the antibiotic genes, at least for actinorhodin and undecylprodigiosin. Additionally, the original *absA* mutation *absA1-542* dramatically reduces the transcription of the actinorhodin pathway-specific activator, *actII-orf4*, and the undecylprodigiosin pathway-specific activator, *redD* (1). In future work, exploration of the spectrum of phenotypes that can result from mutational alteration of the *absA1-A2* genes will help to elucidate the regulatory mechanisms.

An *absA*-encoded signal transduction pathway is likely to be part of a complex interconnected regulatory web. The *actI*::*xylE* fusion was expressed prematurely and reached a higher level in C420 than in J1501. But temporal regulation was not entirely lost, suggesting that additional regulatory elements can influence the transcription of the antibiotic biosynthetic loci. The existence of such a network of regulatory influences has been hinted at in other work. Other genes known to impact *S. coelicolor* antibiotic biosynthesis were discovered because of the observation that their overexpression increased actinorhodin and undecylprodigiosin production. These include *afsQ1-Q2*, which is predicted to encode an additional two-component system (33), and the *afsR-K-R2* locus, which encodes a serine-threonine kinase, a target phosphorylated protein (32, 46), and a protein with some sigma factor domains (66). Either multiple copies of the response regulator *afsQ1* (33) or cloned regions from the *afsR-K-R2* locus (12) restore actinorhodin and undecylprodigiosin production to the *absA* mutant strain C542; the mechanism of this suppression is not known. At least for the case of the *afsR-K-R2*-mediated suppression, it is clear that the *absA542* blockage of the two other characterized antibiotics, CDA and methylenomycin, is not reversed (12). The suppressive effect of cloned copies of these genes suggests functional intersection with the *absA1-A2* pathway. Characterization of the suppressive clone pWC3146, identified in this study, may reveal another regulatory element that interacts with an *absA* signal transduction pathway.

The production of secondary metabolites is usually associated with the transition to stationary phase in eubacteria, so it is not surprising to discover that *S. coelicolor* uses signal transduction pathways to coordinate the expression of these regulons in response to their rapidly changing environments. In the last few years several two-component signal transduction pathways have been shown to be involved in the regulation of secondary metabolism in other bacteria. Examples include *B. subtilis*, which uses the *comA-P* and *degS-U* systems to regulate expression of the antibiotic surfactin, hydrolytic enzymes, and competence genes (37); *Pseudomonas fluorescens* CHA0, which uses the *gacA* response regulator to globally regulate secondary metabolism (39); and *P. fluorescens* Pf-5, which uses *apdA* signal transduction system to regulate antibiotic production (17). In another streptomycete, *Streptomyces peucetius*, the regulator DnrN regulates production of daunorubicin (51); a cognate sensor kinase has not yet been identified. In contrast to AbsA-mediated global regulation, DnrN-mediated regulation is thus far known to affect production of only daunorubicin.

AbsA1 contains a hydrophobic N terminus, suggesting that it responds to an external signal and that the production of antibiotics in *S. coelicolor* is intimately tied to the external environment of the colony. As yet there is no evidence as to what constitutes the signal molecule for an *absA* signal transduction pathway. Some clues regarding the nature of the signaling molecule to which AbsA1 responds may come from the signal transduction pathways involved involved in the regulation of secondary metabolites in other bacteria. For example, the *B. subtilis* ComP and ComA proteins are thought to sense and transduce information regarding cell density. At least two oligopeptides are major components of an extracellular signal, and ComP senses one of these, perhaps directly (42). A cell density signal is also important in antibiotic regulation in *Erwinia* and *Serratia* spp., which can produce the β-lactam compound carbapenem (5, 6). In these bacteria, molecules of an *N*-acyl homoserine lactone structure act as autoinducers to signal cell density (5). In these regulatory systems the sensing proteins are not two-component type systems but rather are homologs of the LuxR protein (47), which was discovered as a regulator of luciferase expression in luminescent marine bacteria (e.g., *Photobacterium fischeri* [formerly *Vibrio fischeri* [21]). Homoserine lactone molecules also function in the sporulation and antibiotic production of various *Streptomyces* species (31), but no evidence links these to *absA* signaling.

It has long been speculated that a greater understanding of the regulation of antibiotic synthesis would eventually lead to improvements in antibiotic yield (13). The isolation and characterization of some of the regulatory elements from *S. coelicolor* have already made some of those goals possible. Further study of these regulatory mechanisms will lead not only to a greater understanding of the initiation of secondary metabolism in *S. coelicolor* but also undoubtedly to the rational genetic design of actinomycetes that overproduce commercially important compounds.

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