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A Putative Two-Component Regulatory System Involved in Secondary Metabolism in *Streptomyces* spp.

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A DNA fragment stimulating actinorhodin, undecylprodigiosin, and A-factor production in *Streptomyces lividans* 66 was cloned from *Streptomyces coelicolor* A3(2). Nucleotide sequencing revealed the presence of an open reading frame of 225 codons, named *afsQ1*, that showed great similarity in amino acid sequence to the response regulators of typical prokaryotic two-component regulatory systems responsible for adaptive responses. The termination codon, TGA, of *afsQ1* overlapped the initiation codon, GTG, of a second open reading frame, *afsQ2*, of 535 codons. The *afsQ2* gene product showed homology with the sensory histidine protein kinases of two-component systems. In agreement with the assumption that the AfsQ1 and AfsQ2 proteins comprise an aspartate-histidine phosphotransfer system, an amino acid replacement from Asp to Glu at residue 52 of AfsQ1, generated by site-directed mutagenesis, resulted in loss of the protein's ability to stimulate antibiotic production in *S. lividans*. Primer extension experiments indicated that transcription of the *afsQ1* and *afsQ2* genes initiates at the translational start codon (GTG) of the *afsQ1* gene. The *afsQ1* and *afsQ2* genes were physically mapped at a chromosomal position near the actinorhodin biosynthetic gene cluster (*act*) by hybridization to Southern blots of restriction fragments separated by pulsed-field gel electrophoresis. Disruption of either *afsQ1* or *afsQ2* on the *S. coelicolor* chromosome by use of phage φC31KC515 led to no detectable change in secondary metabolite formation or morphogenesis. The *afsQ1* gene on pIJ922 suppressed the *S. coelicolor absA* mutation and caused actinorhodin production but did not suppress the *absB* mutation. Southern blot hybridization showed that sequences homologous to *afsQ1* and *afsQ2* are present in almost all of the actinomycetes examined.

Production of secondary metabolites in actinomycetes is genetically controlled by regulatory genes, some closely linked and some not closely linked to the biosynthetic genes. One of the latter is the *afsR* system that globally influences secondary metabolism in *Streptomyces coelicolor* A3(2) (21, 36). Recent work from our laboratory has revealed that the AfsR protein is phosphorylated by a membrane-bound phosphokinase and that the extent of phosphorylation of AfsR probably modulates expression of its target gene(s), thus leading to stimulation of expression of antibiotic production genes (12). The *afsR* gene was identified on a DNA fragment that conferred the ability to produce A-factor and the pigmented antibiotics actinorhodin and undecylprodigiosin on *Streptomyces lividans*, which does not produce these metabolites under the cultural conditions used (17, 19). Several other pleiotropically acting *S. coelicolor* genes that affect production of the secondary metabolites of the strain, including *absA* (1), *absB* (2), and *abaA* (8), as well as many *bld* genes that are needed for both antibiotic production and sporulation (7, 13), have been identified.

We have recently cloned an additional gene from *S. coelicolor* A3(2) by the strategy used for cloning the *afsR* and *abaA* genes. The cloned gene, named *afsQ1*, conferred on *S. lividans* phenotypes similar to those of *afsR* when introduced on a plasmid. The cloned gene is designated *afs* (A-factor synthesis) because, like *afsR*, it regulates production of A-factor in addition to that of the pigmented antibiotics. Here we describe the cloning, nucleotide sequence, transcriptional analysis, and physical mapping on the chromosome of the *afsQ1* gene as well as a second gene, *afsQ2*, located just downstream from *afsQ1*. The great similarity of the amino acid sequences of AfsQ1 and AfsQ2 to those of the response regulators and sensory histidine protein kinases (37), respectively, of typical prokaryotic two-component regulatory systems strongly suggests that the AfsQ1 and AfsQ2 proteins comprise a similar two-component system probably involved in secondary metabolism.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. lividans* 66 HH21 (19) is an A-factor-deficient mutant derived spontaneously from strain TK21. *S. coelicolor* A3(2) M130 (*hisA1 uraA1 strA1* SCP1$^-$ SCP2$^-$) (4) was the source of the *afsQ1* and *afsQ2* genes. *S. coelicolor* M145 (SCP1$^-$ SCP2$^-$) (16) was used for pulsed-field gel electrophoresis. *S. coelicolor* C505 (*absA505*) (2) and C120 (*absB120*) (1), derived from J1501 (*hisA1 uraA1 strA1* Pgl$^-$ SCP$^-$ SCP2$^-$), were obtained from W. Champness. An A-factor-deficient mutant strain, *Streptomyces griseus* FT-1 no. 2 (11), and *Bacillus subtilis* ATCC 6633 as the indicator strain were used for A-factor assays. Other actinomycete strains were obtained from the culture collection of the Institute of Fermentation, Osaka, Japan (IFO). Plasmids pIJ41 (carrying thiostrepton and neomycin resistance) with a copy number of 3 to 4 per genome (16) and pIJ922 (thiostrepton resistance) with a copy number of 1 per genome (16) were used as cloning vectors. Plasmid pIJ2303 (pIJ922 carrying the entire actinorhodin biosynthetic gene cluster [29]) was used as a source of the *act* genes for

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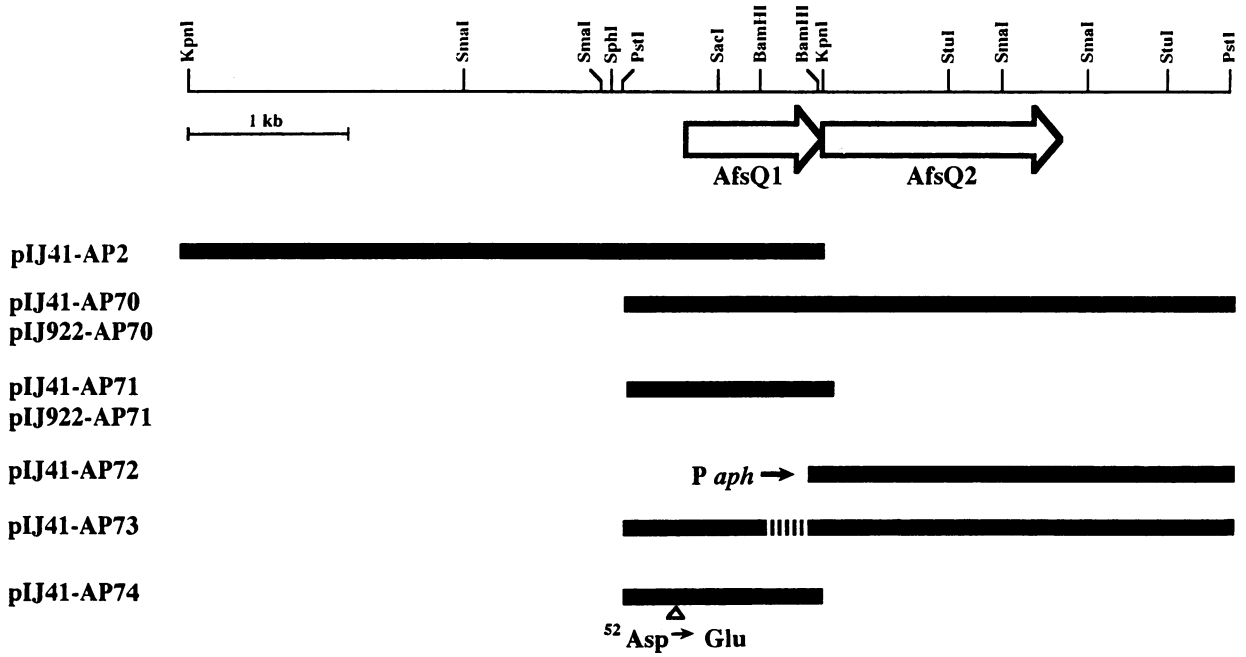

FIG. 1. Restriction map of the DNA fragment containing the *afsQ1* and *afsQ2* genes. The locations of the open reading frames of AfsQ1 and AfsQ2 are indicated by arrows. pIJ41-AP2 was obtained by cloning *Kpn*I-digested chromosomal DNA from *S. coelicolor* M130 into the *Kpn*I site of pIJ41. The 3.8-kb *Pst*I fragment on pIJ41-AP70 was first cloned on pUC19 by colony hybridization and then transferred into the *Pst*I site of pIJ41. For construction of pIJ41-AP71, the 4.3-kb *Kpn*I fragment containing the whole *afsQ1* gene was first inserted into the polylinker of pUC19, and the resulting plasmid was digested with *Pst*I to obtain a 1.25-kb *Pst*I fragment containing the intact *afsQ1* gene. The 1.25-kb *Pst*I fragment was then inserted into the *Pst*I sites of pIJ41 and pIJ922, resulting in pIJ41-AP71 and pIJ922-AP71, respectively. For constructing pIJ41-AP72, the *Bam*HI-*Pst*I fragment containing the whole *afsQ2* gene was first cloned into the polylinker of pUC19, and the resulting plasmid was cut with *Bam*HI plus *Sph*I to obtain a *Bam*HI-*Sph*I fragment containing the intact *afsQ2* gene. The *Bam*HI-*Sph*I fragment was then inserted into pIJ41 digested with *Bam*HI plus *Sph*I. In this construction, the promoter (P*aph*) of the neomycin phosphotransferase gene on pIJ41 is located in front of the *afsQ2* coding sequence, as indicated. pIJ41-AP73, constructed by digestion of pIJ41-AP70 with *Bam*HI and subsequent religation, contains a truncated *afsQ1* gene and intact *afsQ2* gene. pIJ41-AP74 contains a mutated *afsQ1* gene in which Asp-52 is replaced with Glu.

Southern hybridization experiments. For site-directed mutagenesis, *Escherichia coli* JM109 [Δ(*lac-pro*) *thi-1 endA1 gyrA96 hsdR17 relA1 recA1*/F' *traD36 proAB lacI*^{q} *lacZΔM15*] (40) and CJ236(*dut-1 ung-1 thi-1 relA1*/pCJ105) (31) and phage vector M13mp19 (27) were used. DNA was manipulated in *E. coli* JM109 by cloning on pUC19.

General recombinant DNA techniques. Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, reverse transcriptase, and DNA polymerase were purchased from Takara Shuzo, Co., Ltd., or Boehringer GmbH. [α-^{32}P]dCTP (400 Ci/mmol) for nucleotide sequencing by the M13 dideoxynucleotide method (34) with M13mp18 and M13mp19 (40), [α-^{32}P]dCTP (3,000 Ci/mmol) for the Amersham multiprime DNA labeling system, and [γ-^{32}P]ATP (5,000 Ci/mmol) for end labeling of synthetic oligonucleotides with T4 polynucleotide kinase were purchased from Amersham International. Thiostrepton was a gift from Asahi Chemical Industry, Shizuoka, Japan. Viomycin was provided by M. Nakano. DNA manipulations in *E. coli* were performed as described by Maniatis et al. (30), and those in *Streptomyces* were performed as described by Hopwood et al. (16).

Primer extension. *S. lividans* containing pIJ41-AP71 (Fig. 1) and *S. coelicolor* A3(2) M130 were grown at 28°C for 3 days in 10 ml of YEME medium (pH 7.2) containing the following (grams per liter): yeast extract (Difco), 3; Bacto Peptone (Difco), 5; malt extract (Difco), 3; glucose, 10; sucrose, 340; and $MgCl_2 \cdot 6H_2O$, 0.25. After disruption of mycelial clumps with a glass homogenizer, 1 ml of each was inoculated into 100 ml of fresh YEME medium and grown at 28°C for 2 to 4 days. The mycelium was harvested by centrifugation. Total cellular RNA was prepared as previously described (18).

A 20-mer oligonucleotide, 5'-GGCCCTGGCGGGTCAGG GAG-3', complementary to a sequence in the *afsQ1* gene (nucleotides [nt] 580 to 599 in Fig. 2), was synthesized on an Applied Biosystem 380A DNA synthesizer and 5' end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Twenty micrograms of total cellular RNA and 20 ng of 5'-end-labeled oligonucleotide primer were heated at 80°C for 10 min and then at 42°C for 5 h in 50 μl of 20 mM Tris-HCl (pH 8.3)–200 mM KCl. Reverse transcription with 10 U of avian myeloblastosis virus reverse transcriptase was carried out at 42°C for 1 h in 100 μl of a mixture containing 100 mM Tris-HCl (pH 8.3), 100 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM each deoxyribonucleotide, and 50 μg of actinomycin D per ml essentially as described by Fisher and Wray (9). The extended product was analyzed by electrophoresis on an 8% denaturing polyacrylamide gel. Dideoxynucleotide sequencing reactions primed from the oligonucleotide were electrophoresed in neighboring lanes.

Gene disruption with φC31KC515. For disruption of the chromosomal *afsQ1* gene, a 410-bp *Bal*I-*Nae*I fragment (nt 606 to 1008 in Fig. 2) which is internal to the *afsQ1* coding

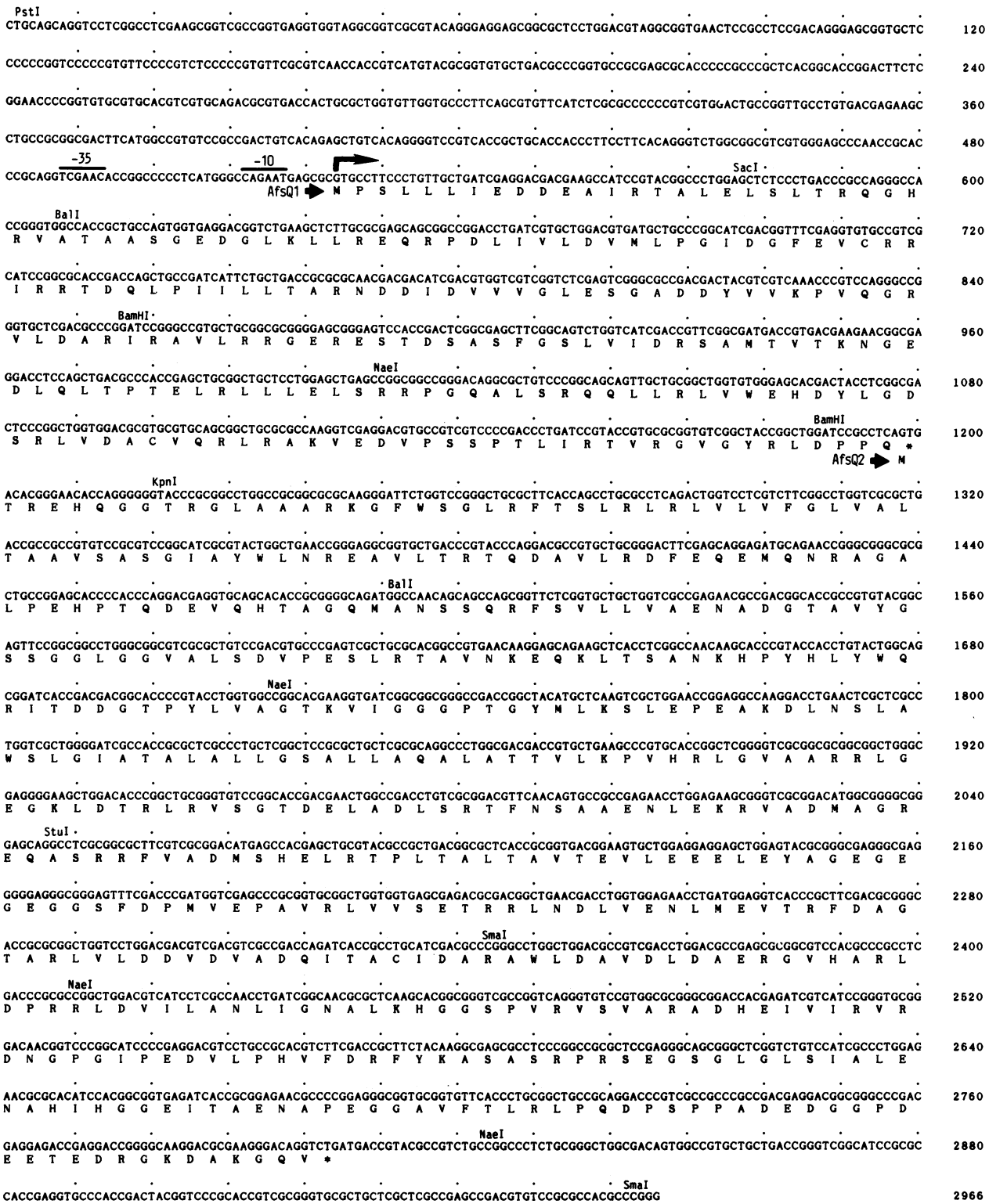

FIG. 2. Nucleotide sequence of the *afsQ1-afsQ2* region and the deduced amino acid sequences of AfsQ1 and AfsQ2. The bent arrow at nt 524 indicates the transcriptional start point, as determined by primer extension. Putative −10 and −35 promoter sequences are overlined.

region was cloned into the *Pvu*II site of a ϕC31 phage vector, ϕC31KC515, in *S. lividans* by liposome-mediated transfection (15). For disruption of the *afsQ2* gene, a *Bal*I-*Sma*I fragment (nt 1489 to 2342) was used. After the *S. lividans* host had been killed by exposure of plates to chloroform vapor for 20 min, the plaques were replicated onto plates spread with spores of *S. coelicolor* M130. After sporulation, viomycin-resistant colonies were selected by replication to

Bennett maltose medium (containing the following [grams per liter]: yeast extract, 1; meat extract [Kyokuto Co.], 1; N.Z. amine [Wako Pure Chemical], 2; and maltose, 10) (pH 7.2) with 10 μg of viomycin per ml. Chromosomal DNA was isolated from one of the colonies in each disruption experiment and analyzed by Southern blot hybridization with the *BalI-NaeI* or *BalI-SmaI* fragment as the probe to confirm integration at an appropriate position on the chromosome.

Site-directed mutagenesis. For generation of an amino acid replacement of residue 52 (GAC, Asp) of the AfsQ1 protein with Glu (GAG), a 26-bp-long nucleotide (5′-GCAGCATC ACCTCCAGCACGATCAGG-3′; italic letters indicate the codon to be replaced) was used. As the target DNA, a 855-bp *PstI-BamHI* fragment (nt 1 to 855 in Fig. 2) covering this region was subcloned into the polylinker of M13mp19. The phage DNA was propagated once in *E. coli* CJ236 to prepare uracil-containing single-stranded DNA (27). The oligonucleotide was annealed with the single-stranded DNA, and the complementary strand was synthesized with DNA polymerase and ligase. *E. coli* JM109 was then transfected with the reaction mixture (41). The mutation thus generated was checked by nucleotide sequencing, and the mutated DNA fragment was transferred to the original *Streptomyces* plasmids, pIJ41-AP70 and pIJ41-AP71.

Assay of A-factor, actinorhodin, and undecylprodigiosin. A-factor production by transformants was assayed by the streptomycin cosynthesis method (11). Briefly, a test strain grown on an agar plug was transferred to a soft agar layer seeded with an A-factor-deficient mutant strain, *S. griseus* FT-1 no. 2, and incubated at 28°C for 2 days. Nutrient soft agar containing spores of *B. subtilis* as the indicator strain was then overlaid, and the plate was further incubated overnight at 37°C. The principle of this method is that A-factor diffusing from the agar plug into the soft agar causes the mutant *S. griseus* strain to produce streptomycin, which in turn is detected by growth inhibition of the indicator. The amount of A-factor was estimated by comparing the diameter of the growth-inhibitory zone with a calibration curve obtained with authentic A-factor and paper disks.

For assay of actinorhodin production, 1 ml of a stationary-phase culture of *S. lividans* or *S. coelicolor* grown in YEME medium was transferred to 100 ml of YMPG medium (containing the following [grams per liter]: yeast extract, 4; Bacto Peptone, 1; malt extract, 10; glucose, 10; and $MgCl_2 \cdot 6H_2O$, 2) (pH 7.2) and incubated at 28°C on a reciprocal shaker. Both media were supplemented with 40 μg of thiostrepton per ml when necessary. A portion (5 ml) was removed at intervals. After removal of mycelium by centrifugation, the A_{615} at pH 12 was measured with a scanning spectrophotometer, using fresh YMPG medium as the reference. Actinorhodin shows its absorption maximum at 615 nm.

For assay of undecylprodigiosin, the pigments were extracted with methanol from mycelium grown at 28°C for 7 days on a cellophane sheet on the surface of Bennett maltose agar medium containing 40 μg of thiostrepton per ml. After concentration of the pigments by evaporation, the orange pigment showing an R_f value of 0.35 was separated by thin-layer (Whatman KC18F reverse-phase plate) chromatography with 100% methanol as the solvent. Undecylprodigiosin shows its absorption maxima at 533 and 468 nm at acidic and alkaline pH, respectively.

Mapping of the *afsQ1* and *afsQ2* genes. Pulsed-field gel electrophoresis of DNA from *S. coelicolor* M145 was carried out by using the LKB Pulsaphor apparatus, incorporating a hexagonal electrode array (catalog no. 2015-100), and digestion by *AseI* as described by Kieser et al. (26). To locate the *afsQ* locus to a specific restriction fragment by Southern hybridization, the probe used was pUC19 carrying a 854-bp fragment extending from the *PstI* site upstream of *afsQ1* to a *BamHI* site internal to *afsQ1* (Fig. 1).

Nucleotide sequence accession number. The nucleotide sequence of the *afsQ1* and *afsQ2* genes has been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence data bases under accession number D10654.

RESULTS

Cloning of the *afsQ1* and *afsQ2* genes. We constructed a bank of *KpnI*-digested fragments of the *S. coelicolor* M130 chromosome, with pIJ41 as the cloning vector, in *S. lividans* HH21. Among approximately 3,000 thiostrepton-resistant *S. lividans* transformants grown on Bennett maltose agar medium, three blue colonies were detected, two of which were found by restriction mapping to harbor a plasmid containing the *afsR* gene. The plasmid from the third transformant contained a 4.3-kb *KpnI* fragment (pIJ41-AP2). The restriction map is shown in Fig. 1. Subcloning experiments showed that a 1.3-kb *PstI-KpnI* fragment on pIJ41-AP71 still conferred actinorhodin production on *S. lividans*. The gene on this fragment was named *afsQ1* because of the characteristics described below.

To isolate DNA downstream from the *afsQ1* gene, we used as a ^{32}P-labeled probe the 0.64-kb *SacI-KpnI* fragment in the *afsQ1* gene for cloning the *PstI* fragment extending rightward from the *PstI* site of pIJ41-AP2. As expected from prior Southern blot hybridization between the *PstI*-digested chromosomal DNA of *S. coelicolor* M130 and the probe, the *PstI* fragment cloned by colony hybridization (10) on pUC19 in *E. coli* JM109 was 3.8 kb in size. We transferred this fragment into the *PstI* site of pIJ41 to obtain pIJ41-AP70. *S. lividans* containing pIJ41-AP70 was judged to produce as much blue pigment as did colonies containing pIJ41-AP2 on Bennett maltose agar medium. The 3.8-kb *PstI* fragment was found to contain the entire *afsQ1* and *afsQ2* genes, as described below.

Nucleotide sequence of the *afsQ1-afsQ2* region. We first determined the nucleotide sequence of the originally cloned 1.3-kb *PstI-KpnI* fragment that was capable of conferring actinorhodin production on *S. lividans*. Computer-aided FRAME analysis (3) predicted a single open reading frame, starting approximately at nt 500 (Fig. 2) and terminating at a TGA codon at nt 1199 to 1201. Although there were two reasonable candidates for the translational start codon, ATG (nt 506 to 508) and GTG (nt 524 to 526), we concluded that translation initiates at the GTG on the basis of the transcriptional initiation site and the alignment of the putative AfsQ1 protein with other regulators of two-component systems (see below).

Because the deduced amino acid sequence of AfsQ1 showed great similarity over the entire sequence to typical regulatory proteins of two-component regulatory systems, we searched for its expected partner gene encoding the corresponding sensor protein on the assumption that a regulatory gene and its sensor gene are often closely linked. As expected, nucleotide sequencing of the region just downstream of *afsQ1* revealed a long open reading frame, named *afsQ2* (Fig. 2), that showed similarity in amino acid sequence to the sensor proteins in two-component regulatory systems (Fig. 3). The TGA termination codon of *afsQ1* overlaps the initiation codon GTG of this open reading frame. This type of overlap strongly suggests that *afsQ1* and *afsQ2* are cotranscribed.

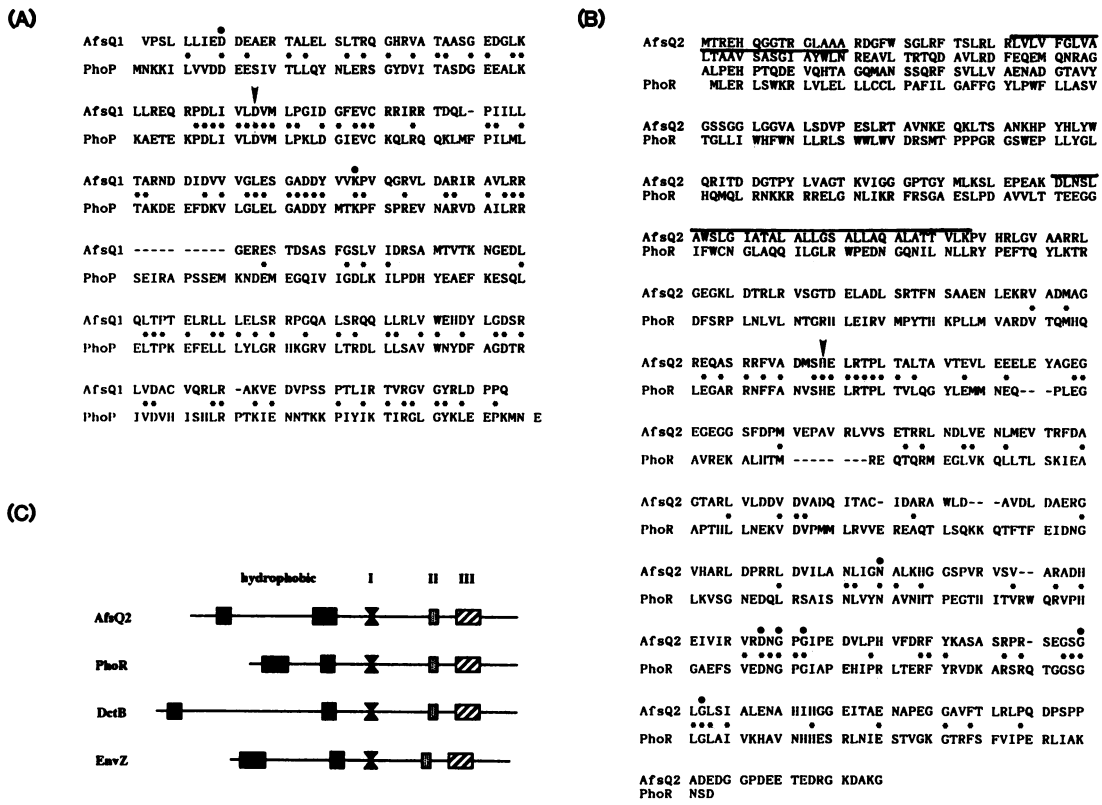

FIG. 3. Alignment of amino acid sequences between AfsQ1 and PhoP (32), a response regulator for phosphate regulation (A), and AfsQ2 and PhoR (28), a histidine protein kinase in the phosphate regulon (B). The putative domain organization of histidine protein kinases is also shown (C). (A) The Asp residue to be phosphorylated is shown by an arrowhead. Asp and Lys residues which tend to be conserved in this family are marked by circles. Identical residues are marked by asterisks. (B) The His residue to be phosphorylated is shown by an arrowhead. Six residues conserved in this family, Asn-(29 residues)-Asp-X-Gly-X-Gly-(24 residues)-Gly-X-Gly (corresponding to regions II and III in panel C) are marked by circles. Identical residues are marked by asterisks. Two hydrophobic putative membrane-spanning sequences of AfsQ2 are indicated by lines over the amino acid sequence. (C) Region I indicates the position of the conserved His residue. Region II contains the conserved Asn residue. Region III contains the Gly-rich sequence. Hydrophobic putative membrane-spanning sequences are indicated by solid boxes near the NH_2 termini.

Sequence similarities of AfsQ1 and AfsQ2 to proteins of prokaryotic two-component regulatory systems. A computer-aided search revealed that AfsQ1 and AfsQ2 resemble the regulators and the sensors, respectively, of two-component regulatory systems (Fig. 3). Prokaryotic two-component systems composed of sensory histidine kinases and response regulators are involved in a range of adaptive responses controlling gene expression in both gram-negative and gram-positive bacteria. A family of the response regulators have an NH_2-terminal domain of similar structure (37) containing an aspartate residue to be phosphorylated. Alignment of AfsQ1 and other regulators allows us to conclude that residue 52 (Asp) is the site of phosphorylation in the AfsQ1 protein. The amino acid sequence of AfsQ1 shows end-to-end similarity to the regulators, including this Asp residue (Fig. 3). In addition, an Asp and a Lys residue (Fig. 3), which tend to be conserved among all regulators, are also conserved in the AfsQ1 protein.

Concerning the AfsQ2 protein as a histidine protein kinase, the His residue to be phosphorylated can be predicted to be residue 294. Within a region of conserved sequence generally located near the COOH terminus in the histidine protein kinase family, there are six residues that are especially conserved: Asn-(15 to 45 residues)-Asp-X-Gly-X-Gly-(20 to 50 residues)-Gly-X-Gly (37). The AfsQ2 protein contains these six residues in the correct order (Fig. 3). The NH_2-terminal portion of AfsQ2 shows no sequence homology with other kinases, consistent with the idea that these portions of the kinases tend to be quite variable. On the other hand, as in many of the histidine kinases, the NH_2-terminal portion of AfsQ2 contains two hydrophobic potential transmembrane sequences bordering a domain that is supposedly localized on the outer surface of the cytoplasmic membrane.

Amino acid replacement at Asp-52 of AfsQ1. As described above, Asp-52 in AfsQ1 was predicted to be phosphorylated. Since phosphorylation of the response regulators of the two-component systems is essential for their regulatory function, we generated an amino acid replacement at Asp-52 by site-directed mutagenesis. In agreement with the prediction, pIJ41-AP74 containing a mutated *afsQ1* gene encoding a protein with a Glu residue at position 52 failed to cause pigmentation in *S. lividans*. These data confirm that Asp-52 of AfsQ1 is essential for the protein's regulatory function and are consistent with this residue being a target for phosphorylation. We could not carry out a comparable replacement of His-294 of AfsQ2 because we had no method of monitoring AfsQ2 activity, since the *afsQ2* gene itself did not cause pigmentation in *S. lividans* (see below).

Transcriptional analysis of the *afsQ1* and *afsQ2* genes.

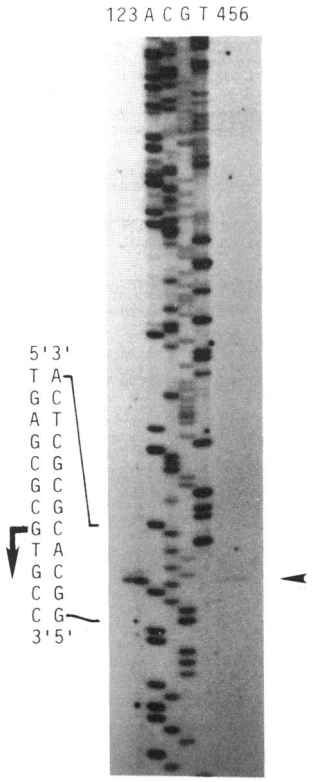

FIG. 4. Identification of the transcriptional start point of the *afsQ1* and *afsQ2* genes by primer extension analysis. RNA was isolated from 2-day (lane 1), 3-day (lane 2), and 4-day (lane 3) cultures of *S. lividans* containing pIJ41-AP71. Primer extension was also done with RNA prepared from 2-day (lane 4), 3-day (lane 5), and 4-day (lane 6) cultures of *S. coelicolor* M130. The sequence ladders, derived from the same primer, are shown with the A, C, G, and T reactions. The arrowhead indicates products of the primer extensions. The arrow indicates the corresponding nucleotide.

Primer extension experiments using a 20-mer synthetic oligonucleotide were used to map the transcriptional start site of *afsQ1*. As shown in Fig. 4, this region had a single transcriptional start point at nt 524 when RNA from *S. lividans* containing pIJ41-AP71 was used. When RNA from *S. coelicolor* M130 was used, a weak signal at the same position was also detected. Upstream of the transcription start point, two hexameric sequences (5′-TCGAAC-3′ and 5′-CAGAAT-3′) separated by 18 nt are present, with some similarity to the *E. coli* consensus −35 and −10 sequences (5′-TTGACA-3′ and 5′-TATAAT-3′, respectively). This type of promoter is believed to be a vegetative promoter in streptomycetes (14). The transcript was detected in the RNA preparation obtained from a 3-day-old culture but not from a 2-day-old culture, and the amount of the transcript was much larger in the RNA from a 4-day-old culture. Under these cultural conditions, *S. lividans* carrying pIJ41-AP2 began to produce actinorhodin 4 to 5 days after inoculation. These observations suggest that active transcription of the *afsQ1* and *afsQ2* genes begins just before actinorhodin production.

Phenotypes conferred on *S. lividans* by the *afsQ1* and *afsQ2* genes. The *afsQ1* gene was cloned by its ability to confer actinorhodin production on *S. lividans*, which is a phenotype similar to that of the *afsR* gene. We examined whether the *afsQ2* gene could also confer pigment production on *S. lividans*. Plasmid pIJ41-AP73 (Fig. 1), containing a truncated *afsQ1* gene and the intact *afsQ2* gene, caused no or little actinorhodin production in *S. lividans*. This finding indicates no significant contribution to pigmentation by the *afsQ2* gene at an increased copy number and is also consistent with the observation that *S. lividans* harboring pIJ41-AP2 (containing only *afsQ1*) and pIJ41-AP70 (containing both *afsQ1* and *afsQ2*) produced almost the same amounts of actinorhodin on Bennett maltose agar medium. The following phenotypes conferred by the *afsQ1-afsQ2* region were therefore ascribable to the *afsQ1* gene.

(i) **Actinorhodin.** *afsQ1* on pIJ41, with a copy number of 3 to 4, caused slight growth inhibition of the *S. lividans* strain, probably because of excessive antibiotic production. This is also true for *afsR* (21). In addition, our repeated attempts to clone the *afsQ1* gene on pIJ101-derived plasmids with a copy number of 40 to 100, such as pIJ385 and pIJ486 (39), were unsuccessful, as in the case of *afsR*. *S. lividans* harboring pIJ922-AP71, with *afsQ1* at a lower copy number, produced a smaller amount of actinorhodin on the agar medium than did the same host harboring pIJ41-AP71. All of these observations suggest that *afsQ1* shows a gene dosage effect. Actinorhodin production of the *S. lividans* transformants in liquid medium was in agreement with that observed on agar medium (Fig. 5A), although *S. lividans* carrying pIJ922-AP71 did not produce a detectable amount of actinorhodin in liquid medium.

(ii) **Undecylprodigiosin.** *S. lividans* harboring pIJ41-AP70 and pIJ41-AP71 produced almost the same amounts of an orange pigment in the mycelium on Bennett maltose agar medium, whereas *S. lividans* harboring only the vector pIJ41 produced little or no such pigment. The absorption spectra of the pigment recovered by thin-layer chromatography showed a sharp peak at 533 nm (orange) at acidic pH and a broad peak at 468 nm (yellow) at alkaline pH (Fig. 5B), indicating that the pigment was undecylprodigiosin. The amount of undecylprodigiosin produced was estimated to be roughly the same as that produced by *S. lividans* harboring *afsR* on pIJ41.

(iii) **A-factor.** In *S. griseus*, A-factor functions as a microbial hormone controlling streptomycin production and resistance and aerial mycelium formation (11, 25). In *S. coelicolor* A3(2) and *S. lividans*, however, A-factor appears to play no such role and is regarded as a secondary metabolite (19, 22). *afsR* on pIJ41 caused production of about 9.5 ng of A-factor per colony in an A-factor-deficient mutant strain, *S. lividans* HH21, when assayed by the streptomycin-cosynthesis method. Similarly, strain HH21 harboring pIJ41-AP70 and pIJ41-AP71 produced about 7.0 ng of A-factor per colony (Fig. 5C).

Chromosomal gene disruption. To examine the role of *afsQ1* and *afsQ2* in secondary metabolism and morphogenesis in *S. coelicolor* A3(2), we disrupted the chromosomal genes by use of phage φC31KC515. The correct disruption of each of the genes was checked by Southern blot hybridization (data not shown). Each of the mutants derived from *S. coelicolor* M130 grew and sporulated normally on agar medium. In addition, both mutants produced actinorhodin, undecylprodigiosin, and A-factor at levels similar to those of the original M130 strain.

Chromosomal location of the *afsQ1-afsQ2* region. A combined physical-genetic chromosomal map has recently been constructed for *S. coelicolor* A3(2) (strain M145) by pulsed-field gel electrophoresis (26). This allowed us to locate the *afsQ* locus on the chromosome by Southern hybridization of a probe, including part of the *afsQ1* gene and upstream

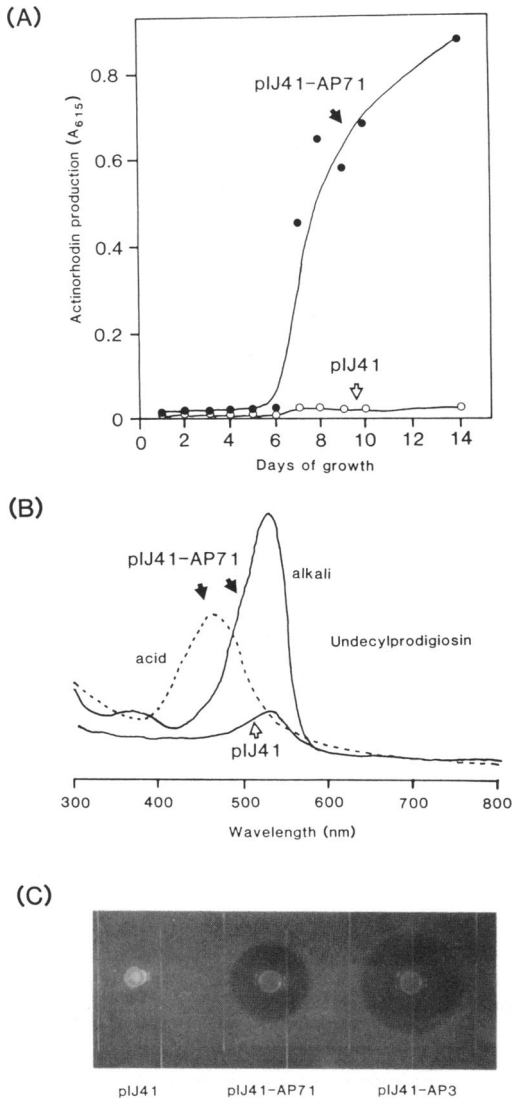

FIG. 5. Production of actinorhodin (A), undecylprodigiosin (B), and A-factor (C) by *S. lividans* HH21 containing *afsQ1*. (A) The A_{615} values of the culture broth, adjusted to pH 12, of *S. lividans* containing pIJ41-AP71 are plotted as a function of cultivation time. Each plot is the mean of the values for three independent experiments. *S. lividans* containing only the vector plasmid pIJ41 produced almost no actinorhodin under these cultural conditions. (B) Undecylprodigiosin was isolated from mycelium by thin-layer chromatography, and its adsorption spectra at pH 12 and 2 were measured. *S. lividans* containing pIJ41 produced no significant amount of undecylprodigiosin. (C) A-factor production was assayed by the streptomycin cosynthesis method with agar plugs on which *S. lividans* transformants were grown. The amount of A-factor produced by the transformant is proportional to the diameter of the growth-inhibitory zone of the indicator strain, *B. subtilis*. An A-factor-deficient mutant, *S. lividans* HH21, containing vector pIJ41 does not produce A-factor, whereas strain HH21 containing the *afsR* gene (pIJ41-AP3) or the *afsQ1* gene (pIJ41-AP71) produces A-factor. The average amounts of A-factor produced by 20 independent colonies containing pIJ41-AP3 and pIJ41-AP71 were 9.5 and 7.0 ng per colony, respectively.

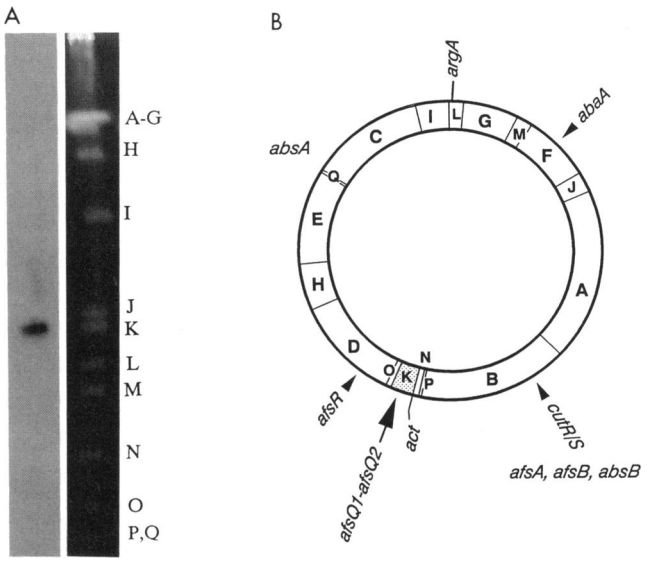

FIG. 6. Mapping of the *afsQ1* and *afsQ2* genes. (A) Southern hybridization of the *afsQ* probe to *Ase*I-digested *S. coelicolor* M145 DNA separated by pulsed-field gel electrophoresis. Note hybridization of the probe to the *Ase*I K fragment. (B) Restriction map of the *S. coelicolor* chromosome showing the arrangement of *Ase*I fragments A to Q and the precise positions of the markers *act* and *argA* as reference points (26). *abaA*, *afsR*, and *cutR/S* were assigned to the approximate positions shown by Southern hybridization to *Ase*I and *Dra*I digests (26). The approximate positions of the *absA*, *absB*, *afsA*, and *afsB* genes are derived from locations on the genetic map and its subsequent alignment with the physical map (26).

sequences, to *Ase*I genomic restriction fragments of strain M145. The probe hybridized to the *Ase*I K fragment (Fig. 6). Of potential relevance, the actinorhodin biosynthetic gene cluster resides partly on the same fragment and partly on the adjacent *Ase*I N fragment (it contains two closely linked *Ase*I sites [26]). To determine whether any of the cloned DNA including *afsQ1-afsQ2* overlapped the cloned *act* DNA, the *Kpn*I fragment in pIJ41-AP2 and the *Pst*I fragment in pIJ41-AP70 were probed against a *Bam*HI digest of pIJ2303 (which carries the entire *act* cluster [29]), but with negative results.

Suppression of *absA* by *afsQ1*. The *absA* and *absB* mutations result in a global block of antibiotic production in *S. coelicolor* (1, 2). *absA* and *absB* were mapped at 10 and 4 o'clock, respectively, on the chromosomal linkage map (Fig. 6). We introduced pIJ922-AP70 and pIJ922-AP72 into *S. coelicolor* C505 (*absA505*) and C102 (*absB102*) to determine whether *afsQ* suppresses the *absA* and *absB* mutations. *S. coelicolor* C505 containing each of the plasmids produced a larger amount of actinorhodin than did its parental strain, J1501, whereas neither of the plasmids caused pigmentation in C102. Thin-layer chromatographic analyses showed that both plasmids conferred the ability to produce undecylprodigiosin on the *absA* mutant. The ability of *afsQ1* to suppress the *absA* mutation is similar to that of *afsR* (2).

Distribution of the *afsQ1* and *afsQ2* sequences among actinomycetes. We examined the distribution of sequences homologous to *afsQ1* and *afsQ2* among actinomycetes by Southern blot hybridization under relatively stringent conditions. The 400-bp *Bal*I-*Nae*I fragment (nt 606 to 1008) for *afsQ1* and the 1,480-bp *Bal*I-*Sma*I fragment (nt 1489 to 2966) for *afsQ2* were used as ^{32}P-labeled probes, and chromosomal

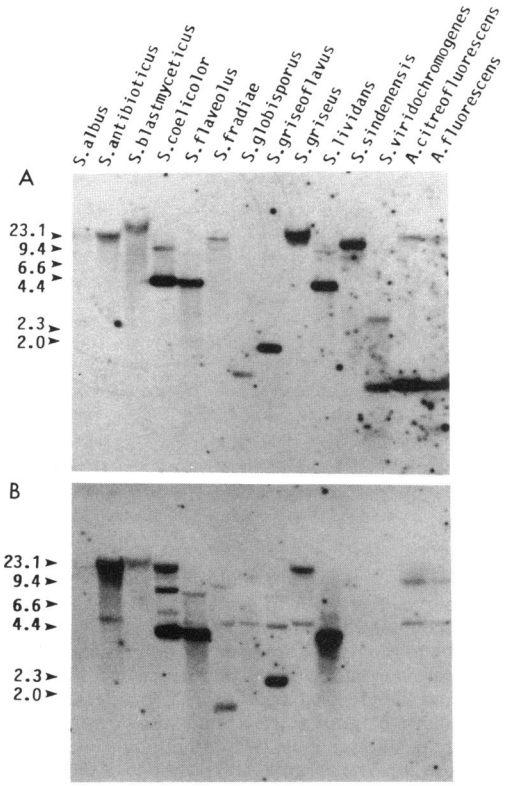

FIG. 7. Distribution of DNA sequences homologous to the *afsQ1* (A) and *afsQ2* (B) probes. The 400-bp *BalI-NaeI* fragment (nt 606 to 1008) for *afsQ1* and the *BalI-SmaI* fragment (nt 1489 to 2966) for *afsQ2* were ^{32}P labeled and used as probes. Chromosomal DNAs digested with *PstI* were used as targets. DNA-DNA hybridization was by the method of Southern (35) at 42°C overnight in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% formamide–0.1% sodium dodecyl sulfate. The nitrocellulose was then washed three times in hybridization buffer at room temperature and twice at 42°C in 0.2× SSC–0.1% sodium dodecyl sulfate. Actinomycete strains examined were (from left to right) *S. albus* IFO 3710, *S. antibioticus* IFO 3126, *S. blastmyceticus* IFO 12747, *S. coelicolor* A3(2) M130 (as a control), *S. flaveolus* IFO 3408, *S. fradiae* ATCC 21096, *S. globisporus* IFO 12208, *S. griseoflavus* IFO 12372, *S. griseus* HH1, *S. lividans* 66 HH21, *S. sindenensis* IFO 12915, *S. viridochromogenes* IFO 12376, *Actinomyces citreofluorescens* IFO 12853, and *A. fluorescens* IFO 12861.

DNAs digested with *PstI* were used as targets. As shown in Fig. 7, DNA sequences homologous to *afsQ1* were found in almost all actinomycetes examined. The strong intensities of the hybridization signals indicate a high degree of similarity. Similarly, sequences homologous to *afsQ2* were found in several of the actinomycetes examined in addition to *S. lividans*. When the 850-bp *BalI-SmaI* fragment (nt 1489 to 2342) for *afsQ2*, which covers the nonconservative region of *afsQ2*, was used as the probe, only *S. antibioticus* and *S. flaveolus*, in addition to *S. lividans*, showed positive signals (data not shown). This finding implies that the nonconservative regions of *afsQ2* homologs are quite variable, as is found for other sensor proteins.

In the *PstI* digests from *S. coelicolor* A3(2) and *S. lividans*, additional weaker signals hybridizing to the *afsQ1* and *afsQ2* probes are seen. It is quite possible that this signal represents genes for another two-component regulatory system.

DISCUSSION

The *afsQ1* gene, which was cloned by its ability to cause actinorhodin production in *S. lividans*, is postulated to be a component of a two-component regulatory system along with the *afsQ2* gene, on the basis of the great similarity of their presumed products, especially in highly conserved regions, to the family of response regulators and histidine protein kinases, respectively. By analogy with the well-characterized two-component systems, such as those for osmoregulation, nitrogen regulation, and phosphate regulation (6, 37), we speculate that the AfsQ2 protein, located in the membrane, is autophosphorylated at His-294 upon sensing a certain environmental signal and then the signal is transferred to the AfsQ1 protein in the cytoplasm by means of phosphotransfer to Asp-52. The phosphorylated form of AfsQ1 would then serve as a transcriptional activator for antibiotic production genes, probably via one or more intermediate regulatory genes. This speculation explains the gene dosage effect of the *afsQ1* gene as the regulator, but not the *afsQ2* gene as the sensor, on the amounts of A-factor and the pigmented antibiotics. Of course, phosphotransfer between AfsQ1 and AfsQ2, and the identity of the target gene(s) of the phospho-AfsQ1, remains to be established, as does the environmental signal for the AfsQ1-AfsQ2 system.

Disruption of the chromosomal *afsQ1* or *afsQ2* gene of *S. coelicolor* A3(2) did not result in any detectable change in secondary metabolite formation or morphogenesis. This finding implies that *afsQ1* and *afsQ2* play no obligatory role in normal antibiotic synthesis in this species but does not exclude a subtle regulatory function for *afsQ1* and *afsQ2*. In *E. coli*, up to 50 signal transduction systems may operate simultaneously (36), and multiple systems similar to the *afsQ1-afsQ2* gene system may also function in a *Streptomyces* strain; thus, effects of mutation in *afsQ1* and *afsQ2* might be quite subtle under the usual laboratory cultural conditions. Suppression of the *absA* mutation by multiple copies of *afsQ1* supports the idea that the *afsQ1-afsQ2* system is involved in regulation of secondary metabolism, dependently on some regulatory genes and independently of other regulatory genes. Clarification of the antibiotic regulation network in *S. coelicolor*, including *afs*, *aba*, *abs*, and *bld*, requires further work and will be useful for a better understanding of antibiotic regulation in other *Streptomyces* spp.

DNA sequences hybridizing to *afsQ1* were found to be widely distributed. Since almost all actinomycete strains examined contained a single hybridizing band in Southern analysis at high stringency, these signals are supposedly counterparts of the *afsQ1* gene. The *Streptomyces* species containing *afsQ1* homologs do not always contain an *afsQ2* homolog (Fig. 7). It is conceivable, as is found for the sensor proteins in other prokaryotic two-component systems, that the AfsQ2 counterparts tend to be more variable than the AfsQ1 regulator proteins.

An additional weaker signal found in *S. coelicolor* A3(2) and *S. lividans* suggests the presence of another sequence homologous to the *afsQ1* gene. Tseng and Chen (38) recently described the CutR-CutS system, which was postulated to regulate copper metabolism in *S. lividans*. They also examined the distribution of *cutR-cutS* homologs by Southern hybridization under conditions essentially similar to those used in our analysis and found only one or two hybridizing fragments in DNA from 16 *Streptomyces* species. Their data suggest that the hybridizing bands represent *cutR-cutS* homologs present in all *Streptomyces* species. The *cutR-cutS* homologs of *S. coelicolor* have recently been mapped to a

position at about 5 o'clock on the physical map (26) and are evidently quite distinct from the *afsQ1* and *afsQ2* genes. We assume that the weak signals detected in *S. coelicolor* A3(2) and *S. lividans* with use of the *afsQ1* probe correspond to a third two-component regulatory system.

Although we have not yet definitively determined the NH_2 terminus of the AfsQ1 product, it appears that a single transcriptional initiation point corresponds to the first nucleotide of the translational start codon. This coincidence of transcriptional and translational start sites has been found in several actinomycete genes, including *ermE*, encoding 23S rRNA methylase (5), *aph*, encoding aminoglycoside phosphotransferase (24), *cat*, encoding chloramphenicol acetyltransferase (33), *sta*, encoding streptothricin acetyltransferase (18), *afsA*, probably encoding an A-factor biosynthetic enzyme (23), and the cholesterol dehydrogenase gene from a *Nocardia* species (20). The ErmE, Aph, and Sta proteins, conferring antibiotic resistance on the hosts, and the AfsA protein, responsible for production of A-factor acting as a trigger of streptomycin production, are supposedly produced just before the production of the respective antibiotics. On the other hand, the cholesterol dehydrogenase was found to be produced from the very early to the late growth phase in *S. lividans*. These observations suggest that this type of unusual transcription-translation coupling is present in a rather wide variety of actinomycete genes.

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REFERENCES

1. **Adamidis, T., and W. Champness.** 1992. Genetic analysis of *absB*, a *Streptomyces coelicolor* locus involved in global antibiotic regulation. J. Bacteriol. **174**:4622–4628.
2. **Adamidis, T., P. Riggle, and W. Champness.** 1990. Mutations in a new *Streptomyces coelicolor* locus which globally block antibiotic synthesis but not sporulation. J. Bacteriol. **172**:2962–2969.
3. **Bibb, M. J., P. R. Findlay, and M. W. Johnson.** 1984. The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequences. Gene **30**:157–166.
4. **Bibb, M. J., and D. A. Hopwood.** 1981. Genetic studies of the fertility plasmid SCP2 and its SCP2* variants in *Streptomyces coelicolor* A3(2). J. Gen. Microbiol. **126**:427–442.
5. **Bibb, M. J., G. R. Janssen, and J. M. Ward.** 1986. Cloning and analysis of the promoter region of the erythromycin resistance gene (*ermE*) of *Streptomyces erythraeus*. Gene **41**:E357–E368.
6. **Bourret, R. B., K. A. Borkovich, and M. I. Simon.** 1991. Signal transduction pathways involving protein phosphorylation in prokaryotes. Annu. Rev. Biochem. **60**:401–441.
7. **Fernández-Moreno, M. A., J. L. Caballero, D. A. Hopwood, and F. Malpartida.** 1991. The *act* cluster contains regulatory and antibiotic export genes, direct targets for translational control by the *bldA* tRNA gene of *Streptomyces*. Cell **66**:769–780.
8. **Fernández-Moreno, M. A., A. J. Martin-Triana, E. Martinez, J. Niemi, H. M. Kieser, D. A. Hopwood, and F. Malpartida.** 1992. *abaA*, a new pleiotropic regulatory locus for antibiotic production in *Streptomyces coelicolor*. J. Bacteriol. **174**:2958–2967.
9. **Fisher, S. H., and L. V. Wray, Jr.** 1989. Regulation of glutamine synthetase gene in *Streptomyces coelicolor*. J. Bacteriol. **171**: 2378–2383.
10. **Grunstein, M., and D. S. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA **72**:3961–3965.
11. **Hara, O., and T. Beppu.** 1982. Mutants blocked in streptomycin production in *Streptomyces griseus*—the role of A-factor. J. Antibiot. **35**:349–358.
12. **Hong, S.-K., M. Kito, T. Beppu, and S. Horinouchi.** 1991. Phosphorylation of the AfsR product, a global regulatory protein for secondary metabolite formation in *Streptomyces coelicolor* A3(2). J. Bacteriol. **173**:2311–2318.
13. **Hopwood, D. A.** 1988. Towards an understanding of gene switching in *Streptomyces*, the basis of sporulation and antibiotic production. Proc. R. Soc. London Ser. B **235**:121–138.
14. **Hopwood, D. A., M. J. Bibb, K. F. Chater, G. R. Janssen, F. Malpartida, and C. P. Smith.** 1986. Regulation of gene expression in antibiotic-producing *Streptomyces*, p. 251–276. *In* I. Booth and C. Higgins (ed.), Regulation of gene expression—25 years on. Cambridge University Press, Cambridge.
15. **Hopwood, D. A., M. J. Bibb, K. F. Chater, and T. Kieser.** 1987. Plasmid and phage vectors for gene cloning and analysis in *Streptomyces*. Methods Enzymol. **153**:116–166.
16. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of *Streptomyces*: a laboratory manual. The John Innes Foundation, Norwich, United Kingdom.
17. **Horinouchi, S., and T. Beppu.** 1984. Production in large quantities of actinorhodin and undecylprodigiosin induced by *afsB* in *Streptomyces lividans*. Agric. Biol. Chem. **48**:2131–2133.
18. **Horinouchi, S., K. Furuya, M. Nishiyama, H. Suzuki, and T. Beppu.** 1987. Nucleotide sequence of the streptothricin acetyltransferase gene from *Streptomyces lavendulae* and its expression in heterologous hosts. J. Bacteriol. **169**:1929–1937.
19. **Horinouchi, S., O. Hara, and T. Beppu.** 1983. Cloning of a pleiotropic gene that positively controls biosynthesis of A-factor, actinorhodin, and prodigiosin in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. J. Bacteriol. **155**:1238–1248.
20. **Horinouchi, S., H. Ishizuka, and T. Beppu.** 1991. Cloning, nucleotide sequence, and transcriptional analysis of the NAD(P)-dependent cholesterol dehydrogenase gene from a *Nocardia* sp. and its hyperexpression in *Streptomyces* spp. Appl. Environ. Microbiol. **57**:1386–1393.
21. **Horinouchi, S., M. Kito, M. Nishiyama, K. Furuya, S.-K. Hong, K. Miyake, and T. Beppu.** 1990. Primary structure of AfsR, a global regulatory protein for secondary metabolite formation in *Streptomyces coelicolor* A3(2). Gene **95**:49–56.
22. **Horinouchi, S., Y. Kumada, and T. Beppu.** 1984. Unstable genetic determinant of A-factor biosynthesis in streptomycin-producing organisms: cloning and characterization. J. Bacteriol. **158**:481–487.
23. **Horinouchi, S., H. Suzuki, M. Nishiyama, and T. Beppu.** 1989. Nucleotide sequence and transcriptional analysis of the *Streptomyces griseus* gene (*afsA*) responsible for A-factor biosynthesis. J. Bacteriol. **171**:1206–1210.
24. **Janssen, G. R., J. M. Ward, and M. J. Bibb.** 1989. Unusual transcriptional and translational features of the aminoglycoside phosphotransferase gene (*aph*) from *Streptomyces fradiae*. Genes Dev. **3**:415–429.
25. **Khokhlov, A. S., I. I. Tovarova, L. N. Borisova, S. A. Pliner, L. A. Shevchenko, E. Y. Kornitskaya, N. S. Ivkina, and I. A. Rapoport.** 1967. A-factor responsible for the biosynthesis of streptomycin by a mutant strain of *Actinomyces streptomycini*. Dokl. Akad. Nauk SSSR **177**:232–235.
26. **Kieser, H. M., T. Kieser, and D. A. Hopwood.** 1992. A combined genetic and physical map of the *Streptomyces coelicolor* A3(2) chromosome. J. Bacteriol. **174**:5496–5507.
27. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154**:367–382.
28. **Makino, K., H. Shinagawa, M. Amemura, and A. Nakata.** 1986. Nucleotide sequence of the *phoR* gene, a regulatory gene for the phosphate regulon of *Escherichia coli*. J. Mol. Biol. **192**:549–556.
29. **Malpartida, F., and D. A. Hopwood.** 1984. Molecular cloning of the whole biosynthetic pathway of a *Streptomyces* antibiotic and its expression in a heterologous host. Nature (London) **309**:462–464.

30. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–78.
32. **Miller, S. I., A. M. Kukral, and J. J. Mekalanos.** 1989. A two-component regulatory system (phoPphoQ) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. USA **86:** 5054–5058.
33. **Murray, I. A., J. A. Gil, D. A. Hopwood, and W. V. Shaw.** 1989. Nucleotide sequence of the chromosomal acetyltransferase gene of *Streptomyces acrimycini*. Gene **85:**283–291.
34. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
35. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
36. **Stein, D., and S. N. Cohen.** 1989. A cloned regulatory gene of *Streptomyces lividans* can suppress the pigment deficiency phenotype of different developmental mutants. J. Bacteriol. **171:**2258–2261.
37. **Stock, J. B., A. J. Ninfa, and A. M. Stock.** 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53:**450–490.
38. **Tseng, H.-C., and C. W. Chen.** 1991. A cloned *ompR*-like gene of *Streptomyces lividans* 66 suppresses defective *melC1*, a putative copper-transfer gene. Mol. Microbiol. **5:**1187–1196.
39. **Ward, J. M., G. R. Janssen, T. Kieser, M. J. Bibb, M. J. Buttner, and M. J. Bibb.** 1986. Construction and characterization of a series of multi-copy promoter-probe plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn*5* as indicator. Mol. Gen. Genet. **203:**468–478.
40. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
41. **Zoller, M. J., and M. Smith.** 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. **100:**468–500.