

# Complex Transcriptional Control of the Antibiotic Regulator *afsS* in *Streptomyces*: PhoP and AfsR Are Overlapping, Competitive Activators<sup>∇</sup>

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The *afsS* gene of several *Streptomyces* species encodes a small sigma factor-like protein that acts as an activator of several pathway-specific regulatory genes (e.g., *actII-ORF4* and *redD* in *Streptomyces coelicolor*). The two pleiotropic regulators AfsR and PhoP bind to overlapping sequences in the  $-35$  region of the *afsS* promoter and control its expression. Using mutated *afsS* promoters containing specific point mutations in the AfsR and PhoP binding sequences, we proved that the overlapping recognition sequences for AfsR and PhoP are displaced by 1 nucleotide. Different nucleotide positions are important for binding of AfsR or PhoP, as shown by electrophoretic mobility shift assays and by reporter studies using the *luxAB* gene coupled to the different promoters. Mutant promoter M5 (with a nucleotide change at position 5 of the consensus box) binds AfsR but not PhoP with high affinity (named “superAfsR”). Expression of the *afsS* gene from this promoter led to overproduction of actinorhodin. Mutant promoter M16 binds PhoP with extremely high affinity (“super-PhoP”). Studies with  $\Delta$ *afsR* and  $\Delta$ *phoP* mutants (lacking AfsR and PhoP, respectively) showed that both global regulators are competitive transcriptional activators of *afsS*. AfsR has greater influence on expression of *afsS* than PhoP, as shown by reverse transcriptase PCR (RT-PCR) and promoter reporter (luciferase) studies. These two high-level regulators appear to integrate different nutritional signals (particularly phosphate limitation sensed by PhoR), *S*-adenosylmethionine, and other still unknown environmental signals (leading to AfsR phosphorylation) for the AfsS-mediated control of biosynthesis of secondary metabolites.

Most of the genes which code for the synthesis of secondary metabolites are arranged in clusters which contain pathway-specific regulators (2, 21). These regulators are frequently controlled by other proteins with global regulatory functions. Examples of these regulators are AfsK, AfsR, and AfsS. These proteins have been shown to influence secondary metabolism and morphogenesis in several species of *Streptomyces* (8, 17, 29, 30, 40, 42).

The complete AfsK/AfsR/AfsS system was first described in *Streptomyces coelicolor* (reviewed in reference 10). AfsK is a loosely attached membrane kinase that autophosphorylates in serine and threonine residues under certain stimuli (e.g., *S*-adenosylmethionine) to enhance its kinase activity (16, 42). The activated AfsK phosphorylates the serine and threonine residues of the AfsR protein (24). Another protein, KbpA, inhibits the autophosphorylation of AfsK (41). Two other serine/threonine kinases, AfsL and PkaG, were also found to phosphorylate AfsR (35), suggesting that AfsR integrates multiple inputs. Phosphorylated AfsR binds the  $-35$  region of the *afsS* promoter and activates its transcription (15, 39). AfsS then stimulates the expression of genes encoding the pathway-specific transcriptional regulators ActII-ORF4 and RedD, which in turn activate the transcription of the actinorhodin and undecylprodigiosin genes (6, 10). Disruption of *afsK*, *afsR*, and

*afsS* genes decreases antibiotic production in *S. coelicolor*, indicating that these gene products act as positive regulators of secondary metabolism in this bacterium (6, 15, 17, 24).

The positive effect which these proteins exert on secondary metabolism has also been observed in other *Streptomyces* species. For example, complementation of a *Streptomyces avermitilis afsK* disrupted mutant with a copy of a plasmid containing the *afsK* gene restored avermectin production (29). Overexpression of the *afsR* gene was shown to increase secondary metabolite production, not only in *S. coelicolor* but also in *S. lividans*, *S. clavuligerus*, *S. griseus*, *S. peucetius*, and *S. venezuelae* (6, 9, 19, 26). A similar effect was produced by the overexpression of the *afsS* gene in *S. coelicolor*, *S. lividans*, *S. avermitilis*, and *S. albus* (6, 14, 20, 25, 43). Therefore, these proteins are described as positive regulators of secondary metabolism in *Streptomyces*.

When the environmental phosphate concentration is low, many bacteria respond through changes in gene expression mediated by a conserved two-component system, named *phoR-phoP* in *Streptomyces*. The response regulator PhoP controls the transcription of *pho* regulon genes in *S. coelicolor* by binding to specific sequences called PHO boxes (37). Interestingly, PhoP binds and regulates the *afsS* gene in competition with AfsR (34). Both AfsR and PhoP regulators recognize overlapping sequences in the promoter region of *afsS*. The role of PhoP in the expression of *afsS* was initially thought to be negative because of its competition with the AfsR activator protein and the higher *afsS* promoter activity obtained in a  $\Delta$ *phoP* mutant than in the wild-type strain. In this study, we prove that both proteins behave as competitive activators of

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TABLE 1. Bacterial strains and plasmids used in this work

Strain/plasmid	Characteristic(s)	Reference or source
<b>Strains</b>		
<i>Streptomyces coelicolor</i> A3(2)		
M145	Wild type	12
INB201	$\Delta$ <i>phoP</i> Am <sup>r</sup>	34
M513	$\Delta$ <i>afsR</i>	6
INB513	$\Delta$ <i>afsR</i> $\Delta$ <i>phoP</i> Am <sup>r</sup>	This work
M952	$\Delta$ <i>afsS</i> Am <sup>r</sup>	Mervyn Bibb
<i>Escherichia coli</i>		
DH5 $\alpha$	F' $\phi$ 80 <i>dlacZ</i> $\Delta$ M15	7
ET12567(pUZ8002)	<i>dam dcm</i> mutant, Neo <sup>r</sup> Cm <sup>r</sup>	18
BL21(DE3)/pLysS	F <sup>-</sup> <i>ompT hsdS<sub>B</sub></i> ( <i>r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup></i> ) <i>gal dcm</i> (DE3)/pLysS (Cam <sup>r</sup> )	Novagen
<b>Plasmids</b>		
pFS- <i>afsS</i>	PCR product carrying <i>afsS</i> promoter cloned into pBSIISK+ <sup>a</sup> , Amp <sup>r</sup>	34
pFS- <i>afsS</i> -M1	PCR product carrying <i>afsS</i> -Mu-0 promoter cloned into pBSIISK+, Amp <sup>r</sup>	This work
pFS- <i>afsS</i> -M2	PCR product carrying <i>afsS</i> -Mu-1 promoter cloned into pBSIISK+, Amp <sup>r</sup>	This work
pFS- <i>afsS</i> -M3	PCR product carrying <i>afsS</i> -Mu-2 promoter cloned into pBSIISK+, Amp <sup>r</sup>	This work
pFS- <i>afsS</i> -M5	PCR product carrying <i>afsS</i> -Mu-4 promoter cloned into pBSIISK+, Amp <sup>r</sup>	This work
pFS- <i>afsS</i> -M14	PCR product carrying <i>afsS</i> -Mu-13 promoter cloned into pBSIISK+, Amp <sup>r</sup>	This work
pFS- <i>afsS</i> -M16	PCR product carrying <i>afsS</i> -Mu-15 promoter cloned into pBSIISK+, Amp <sup>r</sup>	This work
pLUXAR-neo	Integrative promoter-probe vector, <i>luxAB</i> genes, Am <sup>r</sup> Neo <sup>r</sup>	34
pLUX- <i>afsS</i>	BamHI-NdeI pFS- <i>afsS</i> fragment cloned into pLUXAR-neo, Am <sup>r</sup> Neo <sup>r</sup>	34
pLUX- <i>afsS</i> -M1	BamHI-NdeI pFS- <i>afsS</i> -M1 fragment cloned into pLUXAR-neo, Am <sup>r</sup> Neo <sup>r</sup>	This work
pLUX- <i>afsS</i> -M2	BamHI-NdeI pFS- <i>afsS</i> -M2 fragment cloned into pLUXAR-neo, Am <sup>r</sup> Neo <sup>r</sup>	This work
pLUX- <i>afsS</i> -M3	BamHI-NdeI pFS- <i>afsS</i> -M3 fragment cloned into pLUXAR-neo, Am <sup>r</sup> Neo <sup>r</sup>	This work
pLUX- <i>afsS</i> -M5	BamHI-NdeI pFS- <i>afsS</i> -M5 fragment cloned into pLUXAR-neo, Am <sup>r</sup> Neo <sup>r</sup>	This work
pLUX- <i>afsS</i> -M14	BamHI-NdeI pFS- <i>afsS</i> -M14 fragment cloned into pLUXAR-neo, Am <sup>r</sup> Neo <sup>r</sup>	This work
pLUX- <i>afsS</i> -M16	BamHI-NdeI pFS- <i>afsS</i> -M16 fragment cloned into pLUXAR-neo, Am <sup>r</sup> Neo <sup>r</sup>	This work
pBluescriptIISK+	Cloning vector, Amp <sup>r</sup>	Stratagene
pAR- <i>afsS</i>	PCR product carrying complete wild-type <i>afsS</i> gene cloned into pBSIISK+, Amp <sup>r</sup>	This work
pAR- <i>afsS</i> -M5	PCR product carrying complete M5 <i>afsS</i> gene cloned into pBSIISK+, Amp <sup>r</sup>	This work
pAR- <i>afsS</i> -M16	PCR product carrying complete M16 <i>afsS</i> gene cloned into pBSIISK+, Amp <sup>r</sup>	This work
pRA	Conjugative, integrative vector derived from pSET152	28
pARint- <i>afsS</i>	Integrative vector with complete wild-type <i>afsS</i> gene	This work
pARint- <i>afsS</i> -M5	Integrative vector with complete M5 <i>afsS</i> gene	This work
pARint- <i>afsS</i> -M16	Integrative vector with complete M16 <i>afsS</i> gene	This work
pET16- <i>afsR</i> $\Delta$ TTPR	Cloning vector carrying His-tagged truncated AfsR (Met1 to Glu618), Amp <sup>r</sup>	39
pGEX-DBD	<i>S. coelicolor</i> <i>phoP</i> DBD gene cloned into pGEX-2T	37

<sup>a</sup> pBSIISK+, pBluescriptIISK+.

*afsS*, with each being able to activate the transcription of this gene when acting separately. We have identified the nucleotides which are important for the recognition of each protein. Different operators that are able to bind only one, both, or neither of the two proteins have been obtained. The use of one of these mutated operators in the *afsS* gene resulted in an antibiotic-overproducing strain.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** All bacterial strains and plasmids used in this work are listed in Table 1. The *Streptomyces coelicolor* strains used were the parental M145 strain and several mutants derived from it. The  $\Delta$ *afsR*  $\Delta$ *phoP* double mutant (INB513) was obtained from the  $\Delta$ *afsR* mutant strain M513 (6) by replacement of the *phoP* gene with an apramycin resistance cassette as described previously (31). The  $\Delta$ *afsS* mutant M952 was provided by Mervyn Bibb. All of the *Streptomyces* strains were manipulated according to standard procedures (12).

Primers CAR91 (TGAGGAATTCGGAGCCGGTCTCCTGAAC, introduced EcoRI site underlined) and CAR21 (ATGCGGATCCTCAGCCTCTACGAGC AGC, BamHI site underlined) were used to amplify the complete *afsS* gene. After EcoRI-BamHI digestion, the product (578 bp) was cloned into pBluescriptIISK+ to obtain pAR-*afsS*. The insert was sequenced to confirm the absence of mutations. The EcoRV-BamHI fragment was ligated into pRA-neo

vector, a derivative of the conjugative, integrative plasmid pRA (28) containing the kanamycin resistance gene *neo*, to obtain pARint-*afsS*.

All of the *S. coelicolor* cultures were grown in defined MG medium (5) containing 3.2 mM potassium phosphate (MG-3.2 [33]) for phosphate-limited studies.

For luciferase studies, samples were taken at 45 and 50 h of culture, and for antibiotic production assays, samples were collected at 47, 50, 54, 57, 70, and 80 h.

**Production and purification of H-AfsR $\Delta$ TTPR and GST-PhoP<sup>DBD</sup> proteins.** The H-AfsR $\Delta$ TTPR protein (68 kDa; His-tagged protein containing the N-terminal and central regions of AfsR, but with the C-terminal tetratricopeptide repeat [TPR] deleted) (39) was purified from the soluble fraction of *Escherichia coli* BL21(DE3)/pLysS harboring the plasmid pET16-*afsR* $\Delta$ TTPR, as described by Lee et al. (15). The purified samples were dialyzed overnight against a 10 mM Tris-HCl (pH 7)—10% glycerol solution.

The GST-PhoP<sup>DBD</sup> protein (39 kDa; consisting of glutathione *S*-transferase [GST] fused to the DNA-binding domain [DBD] of PhoP) (37) was purified from the soluble fraction of *E. coli* DH5 $\alpha$  harboring the plasmid pGEX-DBD, as described by Sola-Landa et al. (37). Protein concentration was determined by the Bradford method (Bio-Rad).

**Luciferase assay, growth, and antibiotic production.** *luxAB* expression was determined in a Luminoskan luminometer (Labsystems, Helsinki, Finland) as follows. Culture samples were immediately ice cooled; 50  $\mu$ l of 1% *n*-decane was added to 100  $\mu$ l of sample, and the light emission was read after 10 s of integration time. For each culture sample, triplicate measurements were aver-

TABLE 2. Primers used in the SLIM procedure to introduce point mutations in the AfsR and PhoP binding sequences of the *afsS* promoter

Name	Sequence (5'-3')	Strand <sup>a</sup>	Target <sup>b</sup>
AP01	TATCTCCCCTGGCACTG	Dir	DRu-1
AP02	CGGCTACCGCCGGTCGAC	Rev	DRu-1
AP03	GAGTGTTCAGCGTTCGTTTATCTCCCCTGGCACTG	Dir	M1
AP04	AACGAACGCTGAACACTCCGGCTACCGCCGGTCGAC	Rev	M1
AP05	GAGCTTTCAGCGTTCGTTTATCTCCCCTGGCACTG	Dir	M2
AP06	AACGAACGCTGAAAGCTCCGGCTACCGCCGGTCGAC	Rev	M2
AP07	GAGCGATCAGCGTTCGTTTATCTCCCCTGGCACTG	Dir	M3
AP08	AACGAACGCTGATCGCTCCGGCTACCGCCGGTCGAC	Rev	M3
AP09	GAGCGTTTAGCGTTCGTTTATCTCCCCTGGCACTG	Dir	M5
AP10	AACGAACGCTAAACGCTCCGGCTACCGCCGGTCGAC	Rev	M5
AP11	CCTGGCACTGTCATCTCC	Dir	DRu-2
AP12	GAACGCTCCGGCTACCGC	Rev	DRu-2
AP13	AGCGTTCGATTATCTCCCCTGGCACTGTCATCTCC	Dir	M14
AP14	GGGAGATAATCGAACGCTGAACGCTCCGGCTACCGC	Rev	M14
AP15	AGCGTTCGTTTATCTCCCCTGGCACTGTCATCTCC	Dir	M16
AP16	GGGAGATGAACGAACGCTGAACGCTCCGGCTACCGC	Rev	M16

<sup>a</sup> Dir, forward; Rev, reverse.

<sup>b</sup> DRu-1, direct repeat unit 1; DRu-2, direct repeat unit 2.

aged. For dry weight determination, cells were harvested from 2 ml of culture, washed twice with MilliQ water, and dried at 80°C.

Antibiotic production assays were performed as described by Kieser et al. (12).

**Reverse transcriptase PCR (RT-PCR).** RNAs from *S. coelicolor* strains M145, INB201, M513, and INB513 (parental strain and  $\Delta$ *phoP*,  $\Delta$ *afsR*, and  $\Delta$ *afsR*  $\Delta$ *phoP* mutants, respectively) were isolated from 44-h cultures. Commercial kits were used for RNA stabilization in the culture samples (RNA Protect bacterial reagent [Qiagen]), purification (RNeasy minispin columns [Qiagen]), and DNA removal (both an RNase-free DNase set [Qiagen] and a DNA-free kit [Ambion]). RNA concentration and quality were checked using a NanoDrop ND-1000 instrument (Thermo Fisher Scientific) and a 2100 bioanalyzer (Agilent).

Transcriptional analysis of *afsS* was performed using SuperScript one-step RT-PCR with a Platinum *Taq* kit (Invitrogen) and primers *afsS*-Dir-RT (CGA CAAGATGAAGGACGCG) and *afsS*-Rev-RT (TACTTGCCGTCGCCGT CC), which amplified 186 bp of the *afsS* coding region. The primers *hrdB*-Dir-RT (ACGCCCGGCCAGCAGGTC) and *hrdB*-Rev-RT (CAGGTGGCGTACG TGGAGAACTTGT) were used in the control reactions to amplify the *hrdB* transcript (400 bp).

RT-PCR cycling conditions were as follows: 50°C for 60 min and 94°C for 2 min; 10 cycles of 94°C for 30 s and 70°C for 30 s, with a decrease of 1°C per cycle, followed by 72°C for 50 s; 30 to 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 35 s; and, finally, 72°C for 10 min. The optimized reaction mixes contained 200 ng of total RNA, 1.45 mM MgSO<sub>4</sub>, and 5% dimethyl sulfoxide (DMSO). Platinum *Taq* was used in control reactions to ensure the lack of DNA contamination.

**Directed mutagenesis.** Point mutations of the *afsS* promoter were generated with the site-directed ligase independent mutagenesis (SLIM) procedure described by Chiu et al. (4) using the primers detailed in Table 2 and the plasmids pFS-*afsS* (34) and pAR-*afsS* (this work) as templates.

**Electrophoretic mobility shift assays (EMSAs).** The conditions for the DNA-GST-PhoP<sup>DBD</sup> and DNA-H-AfsR $\Delta$ TPR binding reactions were those described by Sola-Landa et al. (37) and Tanaka et al. (39), respectively. Labeling of the DNA fragments was done as described previously (34). A constant DNA concentration was maintained in all reactions. Electrophoresis was carried out in 5% acrylamide gels with 0.5× Tris-borate-EDTA (TBE) buffer for 2 h at 80 V, using a mini-Protean III instrument (Bio-Rad).

**Bioinformatic analysis.** The sequences downstream of *afsR* in the different species of *Streptomyces* were obtained from StrepDB (<http://strepdb.streptomyces.org.uk>) (*S. coelicolor*, *S. avermitilis*, *S. griseus*, and *S. scabiei*) and from GenBank (*S. noursei*). All others were from the Broad Institute database. The sequence corresponding to *S. tsukubaensis* was obtained from the genome sequencing project data at INBIOTEC (unpublished).

The WebLogo page (<http://weblogo.berkeley.edu/logo.cgi>) was used to create the sequence logo of the *afsS* direct repeats.

The scanning of the *S. coelicolor* genome for sequences was done using the RSAT web page (<http://rsat.ulb.ac.be/rsat/>). To calculate the individual informa-

tion content (Ri) of each 11-nucleotide (nt) sequence, the weight matrix of model 1 of PhoP operators was used (38).

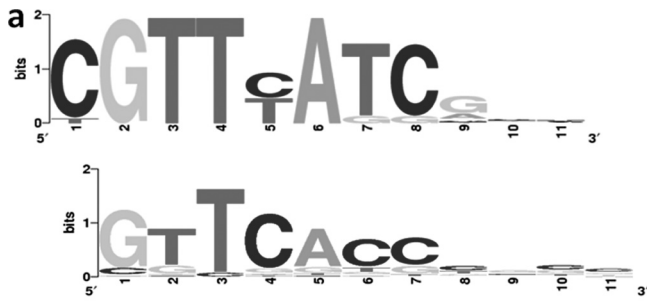
## RESULTS

**Analysis of the PhoP and AfsR binding sequences in the *S. coelicolor afsS* promoter.** Previously, we reported that the PhoP binding site in the *afsS* promoter overlaps with the AfsR binding site in *S. coelicolor* (34). The overlapped sequence encompasses two 11-nucleotide direct repeats. These direct repeats are located 163 nt upstream of the *afsS* start codon and 30 nt downstream of the *afsR* end triplet. Tanaka and coworkers (39) proved the importance of the localization of these repeats with respect to the -10 element and the relevance of both repeat units for the transcription of the *afsS* gene. However, these authors did not determine which nucleotides are necessary for AfsR binding and activation of *afsS* gene transcription.

In order to test the conservation of the AfsR-binding direct repeats among organisms in the *Streptomyces* genus, the *afsS* sequences of 20 different species were analyzed. The alignment of the 40 repeat units showed high conservation in the first 8 nucleotides. This is depicted in the sequence logo shown in Fig. 1a. Nucleotides at positions 2 (G), 3 (T), 4 (T), and 6 (A) are fully conserved in all species analyzed. Positions 1, 7, and 8 are highly conserved (at least 90% of the repeat units share the same nucleotide). Position 5 shows either a C or a T. Interestingly, when the direct repeat units of one promoter are analyzed, each unit has a C or a T, but in no case is the same nucleotide (i.e., C/C or T/T) present at the fifth positions of both repeat units. In summary, the first 8 nucleotides in both repeat units are almost the same among the *Streptomyces* species, and the fifth position shows a fixed pattern of variation that involves both repeats of the binding site. This consensus AfsR-binding sequence is somewhat different from the consensus PhoP binding sequence (Fig. 1a, bottom logo). We decided to analyze by mutagenesis the role of these nucleotides in the regulation of *afsS*.

**Directed modifications of the AfsR and PhoP operators in the *afsS* promoter.** Previously, we described that the binding of





	DRu-1	DRu-2		Total
WT:	(7.0)	(5.8)	CGTTCAGCGTT-CGTTTATCTCC	(12.8)
M1:	(7.0)	(5.8)	tGTTTCAGCGTT-CGTTTATCTCC	(12.8)
M2:	(1.9)	(5.8)	CtTTCAGCGTT-CGTTTATCTCC	(7.7)
M3:	(2.2)	(5.8)	CGTTCAGCGTT-CGTTTATCTCC	(8.0)
M5:	(2.0)	(5.8)	CGTTTAGCGTT-CGTTTATCTCC	(7.8)
M14:	(7.0)	(1.0)	CGTTCAGCGTT-CGATTATCTCC	(8.0)
M16:	(7.0)	(10.8)	CGTTCAGCGTT-CGTTcATCTCC	(17.8)

FIG. 1. (a) (Top) Sequence logo corresponding to the alignment of the 11-nt sequences that form the direct repeat unit of the AfsR and PhoP overlapping operators in the *afsS* gene of the following species of *Streptomyces*: *S. coelicolor*, *S. avermitilis*, *S. lividans*, *S. noursei*, *S. griseus*, *S. scabiei*, *S. tsukubaensis*, *S. clavuligerus*, *S. pristinaespiralis*, *S. roseosporus*, *S. hygroscopicus*, *S. albus*, *S. sviveus*, *S. viridochromogenes*, *S. ghanaensis*, *S. griseoflavus*, *Streptomyces* sp. strain E14, *Streptomyces* sp. strain C, *Streptomyces* sp. strain Mg1, and *Streptomyces* sp. strain SPB74. (Bottom) For comparison, the logo of the PhoP binding sites is shown (33). (b) Point mutations made in the AfsR and PhoP binding sequences of the *S. coelicolor afsS* promoter by SLIM. Lowercase letters indicate the changed nucleotides. The -35 element described by Lee et al. (15) is indicated with shaded boxes, and the conservation value ( $R_i$ ) of each PhoP direct repeat according to model I (38) is shown for each mutant. Note that the PhoP operator starts in the second nucleotide. WT, wild type; DRu-1 and DRu-2, direct repeat units 1 and 2, respectively.

PhoP to the *afsS* promoter caused a negative effect on the expression of *afsS* by hampering the binding of the AfsR protein (the main *afsS* activator) (34). The binding site of PhoP in the *afsS* promoter overlaps the -35 region, and this situation is also found in other genes for which PhoP functions as an activator (22). We introduced point mutations on the *afsS* promoter in order to test whether PhoP is able to activate the expression of *afsS* when AfsR is not present and to elucidate which nucleotides are important for the specific binding of each protein.

To decide which mutations might be more informative, the model of PhoP operators (38) was taken into account. The PhoP binding sites are composed of direct repeat units of 11 nt. The five more frequent nucleotides of the PhoP sequence (GTTCA, positions 1 to 5) match the nucleotides that appear in positions 2 to 6 of the AfsR logo shown in Fig. 1a, with the exception of the T at position 5. By means of an information-based score matrix (model 1 [38]), we were able to predict the effect of mutations on PhoP binding. For each 11-nt repeat, an information content value ( $R_i$ ) was obtained from the matrix.

Using the SLIM procedure (4), we generated 6 point mutations (Fig. 1b). The rationale for these mutations was to discriminate which nucleotides were important for binding of each regulatory protein. In the M1 sequence, the first nucleotide was mutated because it is conserved in the direct repeat of

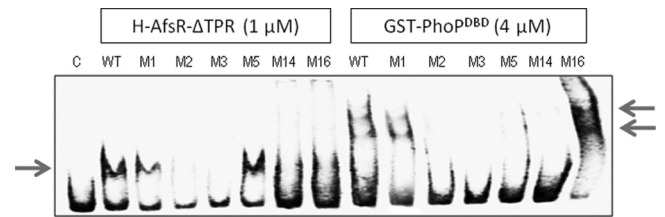


FIG. 2. EMSAs of the wild-type and mutated *afsS* promoters, using the proteins H-AfsRΔTPR and GST-PhoP<sup>DBD</sup>. In lane C (control, using the wild-type promoter), no proteins were added. Note the increase of the PhoP binding activity in the M16 mutant. Note also the cooperative binding of PhoP that results in the formation of several DNA/protein complexes.

*afsS* but it is not conserved in the PhoP operators (so the  $R_i$  value of the PhoP operator did not change) (Fig. 1a and b). For the mutation of the second position (M2), the guanine, although it is always present in the *afsS* repeats, it is conserved in the PhoP operator. As shown in Fig. 1b, the M2 mutation decreases the  $R_i$  value of the mutant operator.

Similarly, with M3, we tested the effect of changing an invariable nucleotide included in the -35 element. In order to determine whether the effect of the mutation was due to the modification of the -35 element, the same mutation was made in the second repeat (M14). In both cases, the  $R_i$  value of the whole PhoP operator decreased to 8.0.

In M5 and M16, artificial CGTTTA and CGTTCA direct repeat units, respectively, were created. As for the other mutants, the PhoP  $R_i$  value of M5 decreased with respect to the wild-type value. On the contrary, the  $R_i$  value of M16 increased five units. The direct repeat generated in the M5 mutation does not appear in any promoter of the *S. coelicolor* genome. On the other hand, the direct repeat generated in M16 appears in the promoters of SCO1394, SCO6372, and SCO7453. Of these three genes, the first two have been described as members of the *pho* regulon in *S. coelicolor* (38). Therefore, it is expected that the site created in M16 binds PhoP with higher affinity than the sites in other mutants.

**In vitro analysis of the binding of AfsR and PhoP to the mutant *afsS* promoters.** We previously described that an H-AfsRΔTPR protein concentration of 0.5 μM was enough to produce a shift in the *phoRP* and *pstS* promoters (34). In addition, we showed that a GST-PhoP<sup>DBD</sup> concentration of 2 μM was enough to produce a shift in the *afsS* promoter.

In this work, to test the abilities of these proteins to bind the different *afsS* mutated promoters, EMSAs were performed. In all cases, the same amounts of DNA (1 ng) and protein (1 μM for H-AfsRΔTPR and 4 μM for GST-PhoP<sup>DBD</sup>) were used to avoid differences in the DNA/protein ratios.

Figure 2 shows that all of the mutations that decreased the  $R_i$  value of the PhoP operator (M2, M3, M5, and M14 mutations) prevented the binding of PhoP. In contrast, the M16 sequence, with an increased  $R_i$  value, yielded a much higher percentage of probe retardation (i.e., increased binding) than the wild-type or M1 fragment. In the M1 fragment, the mutation did not affect the PhoP binding site core. In summary, it can be concluded that, as expected, there was a good correlation between the calculated  $R_i$  variation for each mutant sequence and the loss or gain of PhoP affinity. Due to the in-

creased PhoP binding to the M16 sequence, we named M16 the “superPhoP” operator.

On the other hand, all of the mutations that change the invariable nucleotides of the repeat (M2, M3, and M14 mutations) prevented the binding of AfsR. This result proved the importance of the 2nd (G) and 3rd (T) positions for the binding of AfsR. The involvement of the less conserved cytosine at position 1 was also reflected in the binding assays (Fig. 2). The most favorable nucleotide at position 5 for AfsR binding was a thymine (the M5 sequence is recognized with at least the same affinity as the wild-type sequence), as opposed to a cytosine (M16), which impaired the AfsR protein binding (Fig. 2).

These results (lack of binding of PhoP to M5 and of AfsR to M16) may explain why, in order to bind both proteins, the homologous *afsS* operators always conserve a C in one repeat and a T in the other.

Moreover, the inability of both PhoP and AfsR to bind M3 and M14, with the equivalent positions changed, indicates the need of the two direct repeat units for the binding of both proteins, in concordance with the observations published by Sola-Landa et al. (38) for PhoP and by Tanaka et al. (39) for AfsR.

**Analysis of *afsS* expression in the  $\Delta$ *phoP*,  $\Delta$ *afsR*, and  $\Delta$ *afsR*  $\Delta$ *phoP* strains.** As described above, the *afsS* promoter region has the ability to be bound at least by the AfsR and PhoP proteins. So far, AfsR is the only known activator of the *afsS* gene; however, significant *afsS* promoter activity was detected in a  $\Delta$ *afsR* strain (34). It has not been determined which regulator activates the *afsS* promoter in the  $\Delta$ *afsR* background or whether PhoP might be this second activator.

To study the effect of the lack of both the AfsR and the PhoP protein on *afsS* expression, an  $\Delta$ *afsR*  $\Delta$ *phoP* double mutant (strain INB513) was constructed as indicated in Materials and Methods. The *afsS* transcription in the wild-type,  $\Delta$ *phoP*,  $\Delta$ *afsR*, and  $\Delta$ *afsR*  $\Delta$ *phoP* backgrounds was studied by RT-PCR. Samples for RNA were taken from 44-h MG-3.2 cultures. This medium is limited in phosphate, and the expression of *pho* genes, as well as growth, antibiotic production, and phosphate consumption, has been determined before (32, 33, 34). The *pho* regulon in *S. coelicolor* is induced when the phosphate in the medium reaches a concentration lower than 0.1 mM. In MG-3.2, this occurs after 44 h of culture (33).

Amplification of the control *hrdB* transcript was clearly observed in all of the RNA samples after 40 cycles. No amplification was detected when the retrotranscriptase was omitted, which indicates a lack of DNA contamination (Fig. 3a). The amplification of the *afsS* transcript obtained after 40 cycles was poor (data not shown). When the number of cycles was increased to 45, good amplification was obtained; the highest intensity of *afsS* transcript corresponded to the wild-type and the  $\Delta$ *phoP* strains, in which the AfsR activator is functional (Fig. 3b). The low but significant *afsS* transcript obtained in the  $\Delta$ *afsR* mutant was in agreement with our previous promoter-probe assays (34). On the other hand, the lack of *afsS* transcript in the double mutant points to an activator role of PhoP (in addition to that of AfsR) in *afsS* transcription.

**In vivo analysis of the effect of the point mutations on *afsS* promoter activity.** In order to determine the effect of the point mutations on promoter activity of *afsS*, the wild-type and mutant promoters were cloned into the promoter-probe vector

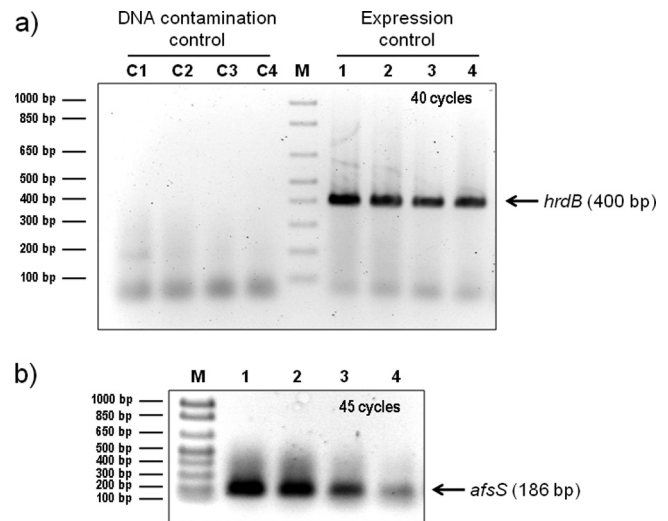


FIG. 3. RT-PCR amplification of the *hrdB* (a) and *afsS* (b) transcripts using the RNA of *S. coelicolor* M145 (parental strain, lane 1), INB201 ( $\Delta$ *phoP* mutant, lane 2), M513 ( $\Delta$ *afsR* mutant, lane 3), and INB513 ( $\Delta$ *afsR*  $\Delta$ *phoP* mutant, lane 4) strains. Cultures were grown in MG-3.2 medium, and samples for RNA were taken at 44 h. The *hrdB* transcript is used as an expression control. For the DNA control reactions (represented by C1 to C4), Platinum *Taq* was added instead of retrotranscriptase. Lane M, molecular size marker.

pLUXAR-neo for comparative studies. The respective plasmids were then introduced into the M145 (wild-type), INB201 ( $\Delta$ *phoP* mutant), M513 ( $\Delta$ *afsR* mutant), and INB513 ( $\Delta$ *afsR*  $\Delta$ *phoP* mutant) strains.

Liquid MG-3.2 defined medium was used to grow the different strains. The maximum promoter activity of *afsS* in this medium is reached after 50 h of growth (34).

The specific luciferase activities of the wild-type *afsS* promoter were lower in the M513 strain ( $\Delta$ *afsR* mutant) and higher in the INB201 strain ( $\Delta$ *phoP* mutant) than in the parental M145 strain at 45 and 50 h (Fig. 4). The almost null promoter activity in the  $\Delta$ *afsR*  $\Delta$ *phoP* background coincided with the results of the RT-PCR experiment.

The specific luciferase activities of all mutant *afsS* promoters which bind neither AfsR nor PhoP (M2, M3, and M14) were significantly reduced compared to that of the wild-type promoter. At the time of maximum *afsS* luciferase activity (50 h), the M2, M3, and M14 values were, respectively, 10%, 7%, and 16% that of the wild-type promoter in the M145 strain and 2%, 3%, and 5% that of the wild-type promoter in the INB201 strain. These results indicate that in M2, M3, and M14 these nucleotide modifications almost abolish the promoter activity of *afsS*, especially in the  $\Delta$ *phoP* mutant strain, confirming that the wild-type nucleotides are important for the AfsR interaction with the promoter. The reduction of *afsS* promoter activity in the promoters that do not bind PhoP and AfsR reveals again a positive role in *afsS* expression not only for AfsR, but also for PhoP.

The specific luciferase activities of the M1 promoter in the M145 strain, which binds both proteins, were similar to those of the parental M145 promoter (85% at 45 h and 77% at 50 h). However, the values in the  $\Delta$ *phoP* (INB201) background were

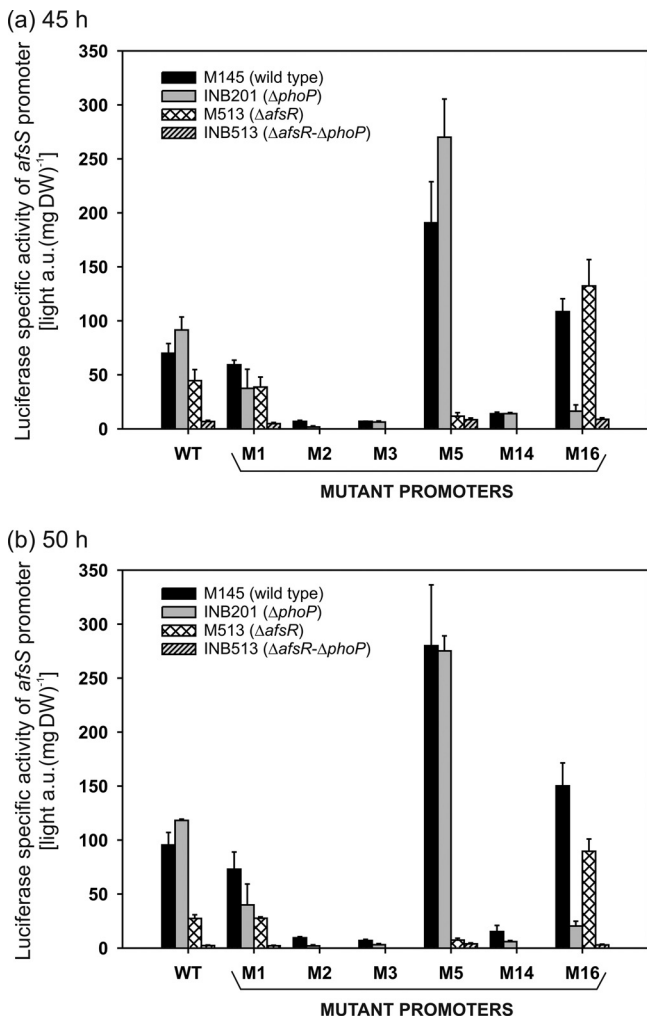


FIG. 4. Promoter activity of *afsS* determined using the *luxAB* reporter of *S. coelicolor* M145 (wild-type), INB201 ( $\Delta phoP$  mutant), M513 ( $\Delta afsR$  mutant), and INB513 ( $\Delta afsR \Delta phoP$  mutant) strains. Each of these strains contained an integrated single copy of pLUX-*afsS*, pLUX-*afsS*-M1, pLUX-*afsS*-M2, pLUX-*afsS*-M3, pLUX-*afsS*-M5, pLUX-*afsS*-M14, and pLUX-*afsS*-M16. The plasmids pLUX-*afsS*-M2, pLUX-*afsS*-M3, and pLUX-*afsS*-M14 were not introduced into strains M513 and INB513, because the low activities obtained with those plasmids in strains M145 (wild type) and INB201 ( $\Delta phoP$  mutant) suggested that no activity would be obtained in strains M513 ( $\Delta afsR$  mutant) and INB513 (double deletion mutant). The cultures were grown in 100 ml of MG-3.2 medium at 30°C and 300 rpm. Samples for dry weight (DW) and luciferase determination were taken at 45 and 50 h of growth (time of maximal expression). Error bars correspond to the standard errors of the means from three biological replicates. a.u., arbitrary units.

less than half of the wild-type promoter activities (41% at 45 h and 34% at 50 h).

On the other hand, in the  $\Delta afsR$  (M513) background, the luciferase values of the M1 promoter were almost equal to those of the wild-type promoter (87% at 45 h and 100% at 50 h). These results indicate that in the M1 promoter the activation due to AfsR, but not the activation due to PhoP, is impaired. This makes sense since the nucleotide C is much more conserved than the nucleotide T in the first position of the direct repeat (Fig. 1a). Moreover, this position is not in-

cluded in the PhoP core operator (28), so it should not be involved in PhoP binding. Our results with the M1 mutant confirmed that the AfsR and PhoP binding sequences start at positions displaced in 1 nucleotide.

With promoters M5 and M16, which bind only one of the two proteins, the luciferase activities are in concordance with the EMSA results. The M5 specific activity values were reduced in the  $\Delta afsR$  strain background (to 26% at 45 h and 27% at 50 h), since it lacks activation by AfsR, but increased in the parental M145 strain (272% and 293% at 45 and 50 h, respectively) and the  $\Delta phoP$  strain (295% and 233% at 45 and 50 h, respectively), where AfsR is functional. This means that the nucleotide T in the fifth position of the direct repeat is required for the activation properties of AfsR and that, as expected, this nucleotide hampers the binding of PhoP (the PhoP operator  $R_i$  value is decreased in M5). Similarly to the M16 sequence (see above), we named the M5 operator “superAfsR.”

On the contrary, the M16 promoter increased the activation properties of PhoP and decreased those of AfsR. Thus, the luciferase values of M16, with respect to those of the wild-type promoter, were 297% at 45 h and 327% at 50 h in the  $\Delta afsR$  strain (where PhoP is the remaining activator) and 18% at 45 h and 17% at 50 h in the  $\Delta phoP$  strain. This result is in agreement with the higher  $R_i$  value of M16 and confirms the “superPhoP” character of this mutant promoter.

For M5 and M16, as for the wild-type and M1 promoters, less than 10% reporter activity values were detected in the  $\Delta afsR \Delta phoP$  double mutant strain with regard to the values for the parental M145 strain. This means that the activation of the *afsS* gene can be exerted by both AfsR and PhoP, with the activation activity of AfsR being higher than that of PhoP. Thus, in the wild-type promoter, when AfsR acts alone (in the  $\Delta phoP$  strain), it generates a higher promoter activity than when both proteins act together (parental M145 strain). Therefore, these proteins may be described as competitive activators (3, 27).

**In vivo analysis of the effect of “superPhoP” and “superAfsR” promoters on antibiotic production.** In order to test whether the high promoter activities obtained with the M5 and M16 promoters in the presence and absence of *afsR*, respectively, affect antibiotic production, the strains M952 ( $\Delta afsS$  mutant) and M513 ( $\Delta afsR$  mutant) were complemented with the pARint-*afsS*, pARint-*afsS*-M5, or pARint-*afsS*-M16 integrative plasmid. The resulting complemented strains, together with the M952 ( $\Delta afsS$  mutant), M513 ( $\Delta afsR$  mutant), and M145 (parental) strains, were grown in MG-3.2 medium and tested for growth and antibiotic production at 47, 50, 54, 57, 70, and 80 h. The introduction into the  $\Delta afsR$  strain of one extra copy of *afsS* with either its own promoter or the “superAfsR” (M5) or “superPhoP” (M16) promoter did not produce a significant effect on the undecylprodigiosin production with respect to that for the  $\Delta afsR$  strain (Fig. 5c). In contrast, the production of actinorhodin was significantly increased in the  $\Delta afsR$  strain with the introduction of the *afsS* gene under the control of the “superPhoP” promoter (Fig. 5b), which is in agreement with its higher promoter activity in this background (Fig. 4). However, the actinorhodin production of this strain (M513/pARint-*afsS*-M16) was still about four times lower than that of the parental strain M145 (Fig. 5b and 6b). Thus, *afsS*



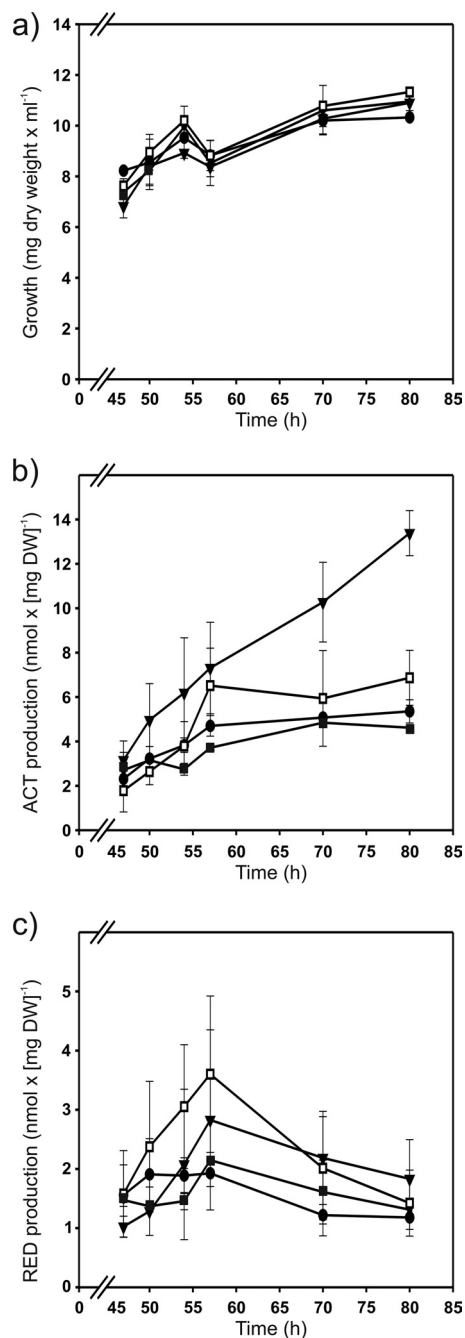


FIG. 5. Growth (a), actinorhodin (ACT) production (b), and undecylprodigiosin (RED) production (c) of *S. coelicolor* M513 ( $\Delta$ *afsR* mutant; white squares) and the complemented strains M513/pARint-*afsS* (black circles), M513/pARint-*afsS*-M5 (black squares), and M513/pARint-*afsS*-M16 (black inverted triangles) grown on MG-3.2. Error bars correspond to the standard errors of the means from three biological replicates.

appears to depend on AfsR for its stimulatory properties, as previously stated by other authors (6). On the other hand, the complementation of the  $\Delta$ *afsS* strain with the *afsS* gene under the control of the wild-type, the “superAfsR” (M5), or the “superPhoP” (M16) promoter restored the wild-type levels of actinorhodin and undecylprodigiosin production (Fig. 6b and

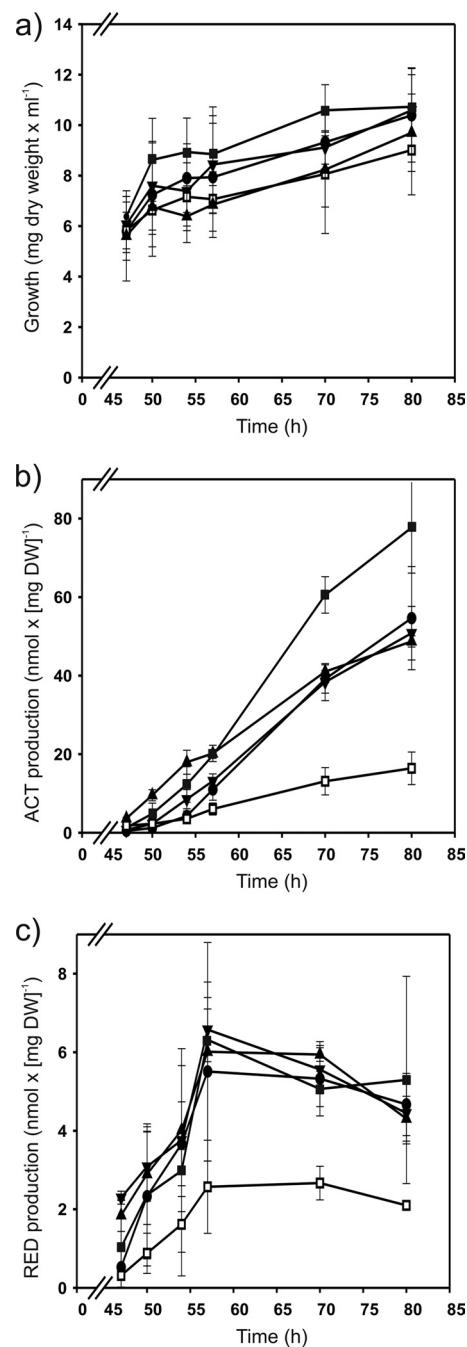


FIG. 6. Growth (a), actinorhodin production (b), and undecylprodigiosin production (c) of *S. coelicolor* M145 (parental strain; black triangles), M952 ( $\Delta$ *afsS* mutant; white squares), and the complemented strains M952/pARint-*afsS* (black circles), M952/pARint-*afsS*-M5 (black squares), and M952/pARint-*afsS*-M16 (black inverted triangles) grown on MG-3.2. Error bars correspond to the standard errors of the means from three biological replicates.

c). As shown in Fig. 6b, the actinorhodin values of the  $\Delta$ *afsS* strain M952 at the times when production rates were highest (at 57, 70, and 80 h) were reduced to 30, 32, and 34%, respectively, of the level of the parental M145 strain. The undecylprodigiosin values were also lower in the  $\Delta$ *afsS* strain (43, 45, and 49% that of the parental strain at 57, 70, and 80 h, respec-

tively [Fig. 6c]). This reduction of antibiotic production in the  $\Delta$ *afsS* strain is in agreement with data published by Lee et al. (15) and Lian et al. (17), although in the latter study, in R5 cultures no actinorhodin production at all was obtained in the *afsS* disruption mutant. The results of our work with MG-3.2 medium show that deletion of *afsS* drastically reduces actinorhodin and undecylprodigiosin production (although it does not suppress it) and that complementation with the gene restores wild-type production levels. Obviously, the antibiotic production in the  $\Delta$ *afsS* mutant is culture medium dependent.

In addition, Fig. 6b shows that complementation of the  $\Delta$ *afsS* mutant with the “superAfsR” (M5) *afsS* gene significantly increases actinorhodin production in comparison with the level achieved by introduction of the native gene or the “superPhoP” *afsS* gene. This result indicates that AfsR is a stronger activator for *afsS* than PhoP. In fact, the actinorhodin production of the strain transformed with the “superAfsR” gene is also higher than that of the wild-type strain (1.5 and 1.6 times higher at 70 and 80 h, respectively). However, as with the “superPhoP” construction in the  $\Delta$ *afsR* strain, no significant differences in undecylprodigiosin production were observed among the different  $\Delta$ *afsS* complemented strains (Fig. 5c and Fig. 6c).

In summary, we conclude that the high activities of the “superAfsR” and “superPhoP” promoters correlate with an increase of actinorhodin production and that, as indicated by other authors, AfsS seems to regulate actinorhodin production to a greater extent than that of undecylprodigiosin, which is also the case with AfsR (6, 15, 17, 34).

## DISCUSSION

Production of antibiotics and other secondary metabolites plays an essential role in bacterial interactions with other living beings; therefore, the expression of the antibiotic biosynthetic genes has to be tightly controlled. The genetic control includes several pathway-specific, broad-range, and global regulators, which in turn respond to different physiological signals, such as phosphate starvation (2, 21). The phosphate starvation response in *Streptomyces* is mediated by PhoP, a transcriptional regulator which belongs to the OmpR family (31, 36, 37). The DNA-binding domain of OmpR consists of three  $\alpha$ -helices packed against two antiparallel  $\beta$ -sheets, forming a winged helix-turn-helix (23).

On the other hand, the wide-range transcriptional regulator AfsR consists of three major functional regions: the 270 N-terminal residues, which have the same domain structure as *Streptomyces* antibiotic regulatory proteins (SARPs); the central ATPase domain; and a C-terminal tetratricopeptide repeat (TPR). SARPs are characterized by a DNA-binding domain that resembles that of OmpR and by an accompanying bacterial transcriptional activation domain, BTAD (39, 44).

Traditionally, the AfsK/AfsR/AfsS system was thought to comprise a linear signal transduction system for secondary metabolism in *Streptomyces* (10). However, in spite of its apparently linear hierarchy, the interaction of these regulators with other regulatory proteins was later reported in *S. coelicolor* (11, 22, 34). AfsR can be phosphorylated by kinases other than AfsK (35), and in turn, AfsR is able to regulate antibiotic

production mediated or not by AfsS (15). Thus, protein binding analyses with purified AfsR showed that AfsR is able to interact with the promoters of genes other than *afsS*, such as *pstS*, *phoRP* (34), and *glnR* (data not shown). Moreover, transcriptomic analyses revealed the implication of AfsS in the regulation not only of genes involved in secondary metabolism but also of genes involved in phosphate, nitrogen, and sulfate assimilation (17). In *S. lividans*, the transcription of *afsS* is modulated by the carbon source of the culture medium (13). In addition, *afsS* can be activated by proteins other than AfsR, as has been shown in a  $\Delta$ *afsR* background (34). There are three *afsR* homologous genes in the *S. coelicolor* genome (1), but only AfsR (which is linked to *afsS*) has been reported to bind the *afsS* promoter gene (15). On the other hand, results from EMSA and reporter (luciferase) studies have demonstrated that PhoP interferes with the action of two different regulators, AfsR (34) and GlnR (32). PhoP, but not GlnR (data not shown), is able to bind to the *afsS* promoter. Overall, it can be concluded that these regulators are involved in a regulatory network mechanism in which multiple interactions between the regulators and other proteins occur.

In this study, using  $\Delta$ *afsR* and  $\Delta$ *phoP* strains and a  $\Delta$ *afsR*  $\Delta$ *phoP* double mutant, in combination with mutant *afsS* promoters that show affinity toward only one or neither of the proteins AfsR and PhoP, we have proven that PhoP is able to activate *afsS* transcription. Thus, almost null *afsS* promoter activity was detected in the  $\Delta$ *afsR*  $\Delta$ *phoP* strain. The same occurred in the mutated *afsS* promoters that were unable to bind either of these two proteins. The highest promoter activities were observed with the promoters that bind only one protein (M5 or M16), with M5 (named “superAfsR”) being the best one. In fact, by improving the PhoP recognition sequence (mutant sequence M16), we observed increased transcription of *afsS* in a  $\Delta$ *afsR* strain. In addition, we found that the greater ability of AfsR to activate *afsS* expression (mutant sequence M5) resulted in an increase of actinorhodin production.

The effects of molecular mechanisms of the *afsS*-encoded protein on the regulation of secondary metabolism are still unknown. Previous reports (6, 15, 17, 34), as well as the results of this work, indicate that AfsS regulates mainly the actinorhodin production in *S. coelicolor*. There is not always a correlation between *afsS* expression and antibiotic production. Thus, the *afsS* promoter activity of the  $\Delta$ *phoP* strain was higher than that of the wild type, but the antibiotic production was lower in this mutant. The same lack of strict correlation was observed when limited and replete phosphate cultures of both wild-type and  $\Delta$ *afsR* strains were compared (34), suggesting alternative control points of the antibiotic pathway. In this study, we have determined the positive correlation between higher *afsS* promoter activities (those of the “superAfsR” and “superPhoP” promoters) and higher actinorhodin production (Fig. 5b and 6b), although the promoter activities and the actinorhodin production were not proportional. The undecylprodigiosin production was not affected.

The main conclusion from this study is that both AfsR and PhoP stimulate *afsS* transcription in a competitive manner, but to different extents, with AfsR being a more potent activator of *afsS* than PhoP. A similar transcriptional regulatory mechanism in *E. coli* was described previously by Peeters et al. (27). These authors demonstrated that the Lrp and ArgP regulators



act as competitive activators on a single promoter, with each regulator being more potent in the absence of the other. The molecular basis of this interference resides in the reciprocal inhibition of Lrp and ArgP binding to overlapping targets in the *argO* promoter region.

Although the *argO* gene of *E. coli* (codes for an L-arginine exporter) and the *afsS* gene of *S. coelicolor* (encoding a small protein involved in secondary metabolism) have different functions, they share almost identical features in their regulation mechanisms, as follows. (i) Both genes are regulated by a specific activator protein (ArgP and AfsR, respectively, for *argO* and *afsS*) and a global regulator which responds to nutritional conditions (Lrp and PhoP, respectively). (ii) ArgP and AfsR form a unique DNA/protein complex in EMSA experiments, while Lrp and PhoP bind cooperatively to multiple binding sites, forming several DNA/protein complexes (Fig. 2). (iii) The binding of ArgP and Lrp to *argO* and the binding of AfsR and PhoP to *afsS* are mutually inhibitory. (iv) Both pairs of regulators (ArgP-Lrp and AfsR-PhoP) behave as competitive activators of *argO* and *afsS*, respectively. (v) The specific activators ArgP and AfsR are more potent activators of *argO* and *afsS* than the global regulators Lrp and PhoP, respectively.

The fact that two very different systems and organisms share similar regulatory mechanisms suggests that the competitive activation of two regulators on a single promoter is a useful mechanism in bacteria, which may serve to integrate different signal inputs on expression of a key regulatory gene without altering too much the proper level of transcription of that regulatory gene. An accumulative rather than competitive effect of the two regulatory proteins would result in an abnormally high expression of *afsS* that might hamper proper cell development in nature.

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