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Cross-talk between two global regulators in *Streptomyces*: PhoP and AfsR interact in the control of *afsS*, *pstS* and *phoRP* transcription

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Summary

The regulatory proteins AfsR and PhoP control expression of the biosynthesis of actinorhodin and undecylprodigiosin in *Streptomyces coelicolor*. Electrophoretic mobility shift assays showed that PhoP^{DBD} does not bind directly to the *act*II-ORF4, *redD* and *atrA* promoters, but it binds to the *afsS* promoter, in a region overlapping with the AfsR operator. DNase I footprinting studies revealed a PhoP protected region of 26 nt (PHO box; two direct repeats of 11 nt) that overlaps with the AfsR binding sequence. Binding experiments indicated a competition between AfsR and PhoP; increasing concentrations of PhoP^{DBD} resulted in the disappearance of the AfsR–DNA complex. Expression studies using the reporter *luxAB* gene coupled to *afsS* promoter showed that PhoP downregulates *afsS* expression probably by a competition with the AfsR activator. Interestingly, AfsR binds to other PhoP-regulated promoters including those of *pstS* (a component of the phosphate transport system) and *phoRP* (encoding the two component system itself). Analysis of the AfsR-protected sequences in each of these promoters allowed us to distinguish the AfsR binding sequence from the overlapping PHO box. The reciprocal regulation of the *phoRP* promoter by AfsR and of *afsS* by PhoP suggests a fine interplay of these regulators on the control of secondary metabolism.

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Introduction

Streptomyces and many other soil-dwelling actinomycetes produce an impressive array of secondary metabolites, many of them having important biological activities (Crandall and Hamill, 1986; Von Döhren and Gräfe, 1997; Martín *et al*., 2000). Nevertheless, very little is known about the regulation of secondary metabolism in *Streptomyces*. Therefore, a better understanding of how regulatory genes act in the secondary metabolism is of great importance. The biosynthesis of antibiotics and other secondary metabolites is controlled by interactions of both global and pathway-specific regulators that trigger, or repress, expression of the genes encoding secondary metabolite biosynthetic enzymes. These regulators respond to a variety of environmental stimuli like stress-inducing agents or nutritional imbalance; i.e. phosphate starvation.

We reported previously that phosphate control of actinorhodin and undecylprodigiosin in *Streptomyces lividans* is mediated by the two-component PhoR–PhoP system (Sola-Landa *et al*., 2003). Purification of the *Streptomyces coelicolor* PhoP response regulator – 100% identical to that of *S. lividans* – allowed the initial characterization of its operators (Sola-Landa *et al*., 2005). These sequences, commonly known as PHO boxes, are formed of 11 nt direct repeat units (DRu). DNA binding of phosphorylated PhoP occurs following phosphate depletion in the culture media and this binding controls the expression of phosphate-regulated genes (Sola-Landa *et al*., 2005; Rodríguez-García *et al*., 2007).

PhoP acts as an activator of genes encoding phosphate scavengers, such as the PstSCAB phosphate transporter system, the alkaline phosphatase PhoA, and two glycerophosphodiesterases (Sola-Landa *et al*., 2005; Apel *et al*., 2007; Rodríguez-García *et al*., 2007). Other genes are repressed by PhoP, such as the *hrdA* sigma factor, the putative oxidoreductase gene SCO2262, and the putative regulator SCO4261 (Sola-Landa *et al*., 2008). Furthermore, the phospholipase *phoD* and the phosphate transporter *pitH2* genes possess complex operators that appear to be subjected to dual phosphate induction and repression mechanisms, depending upon the position of

the DRu with respect to the −35 and −10 regions of their promoters (Apel *et al*., 2007; Santos-Beneit *et al*., 2008; Sola-Landa *et al*., 2008). How does PhoP regulate antibiotic production? It is possible that PhoP interacts with global regulators (AfsR, AtrA), or that it controls cluster-specific regulatory genes (*redD*, *act*II-ORF4), or regulates structural genes directly (Martín, 2004).

The biosynthesis of actinorhodin and undecylprodigiosin is controlled by the global regulator AfsR, a member of the large-size SARP family (Horinouchi *et al*., 1983). Floriano and Bibb (1996) found that the stimulatory effect of AfsR on antibiotic production is exerted through the pathway-specific regulatory genes *act*II-ORF4 and *redD*. Nevertheless, the only known target of the AfsR regulator is a small gene located downstream of *afsR,* named *afsS* in *S. coelicolor* and *afsR2* in *S. lividans*. Amplification of *afsR2/afsS* on a high-copy-number plasmid conferred overproduction of actinorhodin and undecylprodigiosin in both *S. coelicolor* and *S. lividans* (Vögtli *et al*., 1994; Matsumoto *et al*., 1995; Floriano and Bibb, 1996). The binding sequence of AfsR to the *afsS* promoter region was identified by Lee *et al*. (2002); our analysis of this sequence identified a putative PHO box overlapping with the AfsR binding sequence suggesting that both PhoP and AfsR might interact in the regulation of *afsS*. This work demonstrates that both proteins compete for overlapping sequences in the *afsS* promoter, providing the first description of a secondary metabolism regulatory gene controlled by PhoP. In addition, AfsR turned out to regulate the *PhoR–PhoP* system and to repress the *pstS* gene, a major PHO regulon member involved in phosphate transport.

Results

Growth and antibiotic production in wild-type, ΔphoP*, and* ΔafsR S. coelicolor *strains*

Streptomyces coelicolor A3(2) produces several unrelated antibiotics, two of which are easily identified thanks to their colour. In liquid cultures, the red colour is caused by the cell-bound accumulation of undecylprodigiosin and other prodigionines (Tsao *et al*., 1985). At later times, cultures become blue due to the synthesis of actinorhodin, which is linked to the cells, and to its lactone form (γ-actinorhodin) that is exported to the medium (Bystrykh *et al*., 1996). We will refer to the mixtures of red and blue antibiotics as Red and Act respectively.

It is well known that inorganic phosphate (Pi) exerts a negative control on the biosynthesis of a great variety of antibiotics and other secondary metabolites (Martín, 2004). The published works on *S. coelicolor* physiology have limited their focus to Act (Doull and Vining, 1990; Ozergin-Ulgen and Mavituna, 1993; Bystrykh *et al*., 1996; Melzoch *et al*., 1997), but there is very limited information on phosphate control of Red biosynthesis (Hobbs *et al*., 1990). These authors reported the inhibitory effect of Pi on both Act and Red final yields, measured after 72 h of culture. Besides, our laboratory showed the positive effect of the *phoP* deletion on the Act and Red productions in *S. lividans* (Sola-Landa *et al*., 2003). Following these studies, we decided to test the effect of *phoP* and phosphate on antibiotic production in *S. coelicolor* cultures. For this we constructed a new Δ*phoP* mutant, INB201, derived from *S. coelicolor* M145(a plasmid-cured derivative of the wild-type A3(2) strain), as described in *Experimental procedures*. We included also in this study the Δ*afsR* mutant strain M513 (Floriano and Bibb, 1996). These strains were cultured in liquid MG, a defined medium used in previous works (Doull and Vining, 1989; Rodríguez-García *et al*., 2007; Santos-Beneit *et al*., 2008), supplemented with 3.2 mM Pi (MG-3.2) or with 18.5 mM Pi (MG-18.5). In MG-3.2, Pi is depleted (< 0.1 mM) from the medium at 45 h, while it is always in excess in MG-18.5 (Santos-Beneit *et al*., 2008).

Growth of the Δ*phoP* mutant was impaired in the Pi-limited condition (Fig. 1A), as described previously (Santos-Beneit *et al*., 2008). In contrast, the Δ*phoP* mutant and the parental strain growth curves overlapped in the Pi-replete medium. The M513 Δ*afsR* mutant growth was slightly higher throughout the entire time-course than that of the parental M145 strain, especially under Pi limitation (Fig. 1A). Floriano and Bibb (1996) reported that overexpression of *afsR* on strains grown on solid media caused smaller colonies, but these authors attributed this effect to the higher antibiotic production rather than to a deleterious effect of AfsR.

The Red and Act synthesis in the wild-type strain initiated during the transition phase (45–50 h; Fig. 1A–C). Act production in phosphate-replete MG-18.5 was reduced to 50% of that in MG-3.2, either comparing final Act titres or production rates (calculated from the slope of the three last time points; Fig. 1B). Red production was severely inhibited by the high Pi concentration (down to 4%, mean value of 50–75 h; Fig. 1C).

The deletion of *phoP* resulted in a lower and delayed production of both antibiotics under Pi limitation. The Act levels at 50 h, 55 h and 75 h were reduced to 12%, 10% and 51% of the levels in the parental strain; and Red levels decreased to 18%, 24% and 54% respectively.

Deletion of *afsR* affected more severely Act production (11% of that of the parental strain, mean value of 50–75 h) than Red production (51%, mean of 50–75 h) and did not delay the onset of Red in the Pi-limited condition. The Δ*afsR* strain cultures in Pi-replete MG-18.5 had strongly reduced antibiotic production; only residual Act levels

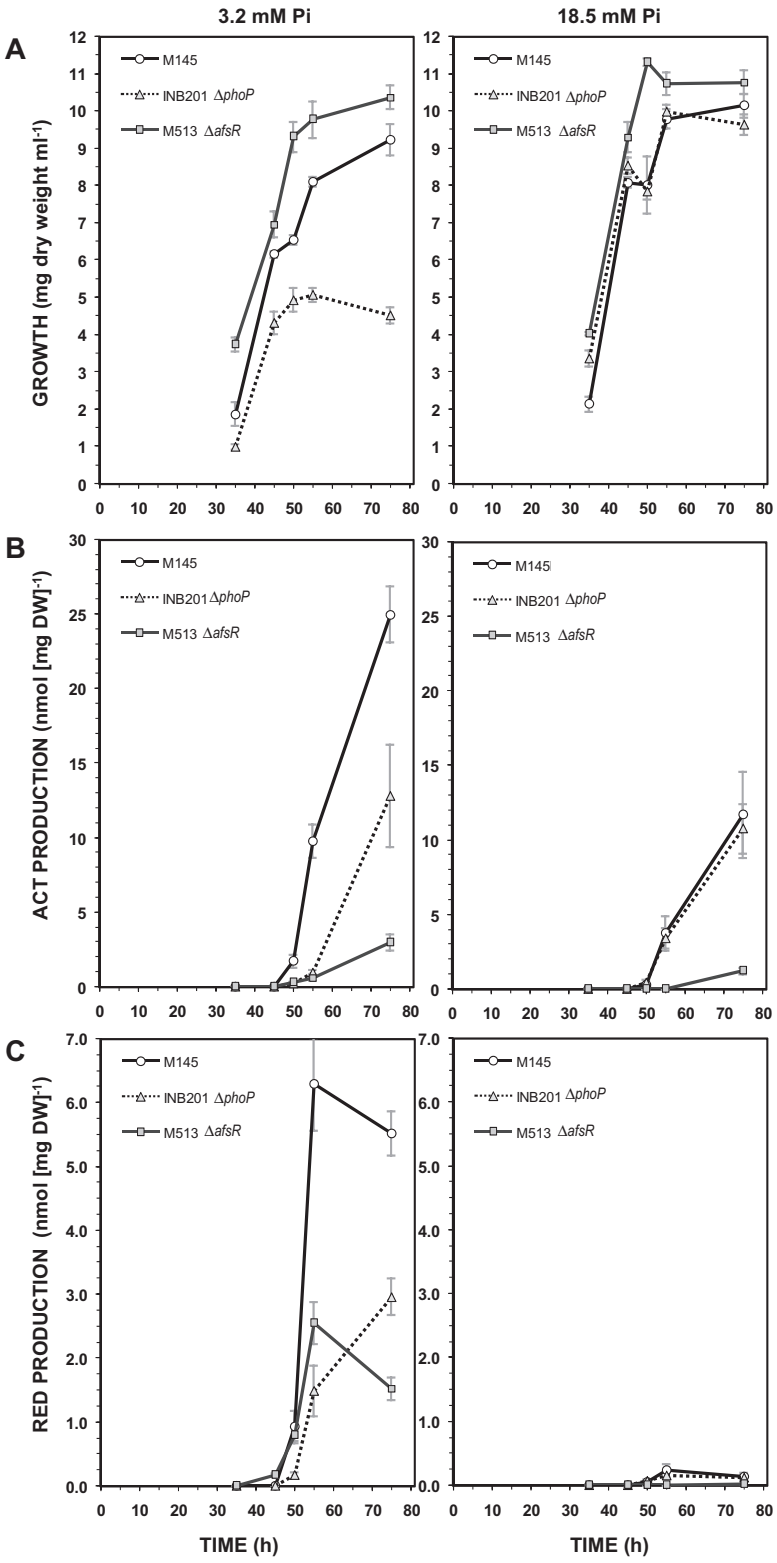

Fig. 1. Growth (A), Act production (B) and Red production (C) of *S. coelicolor* strains M145 (parental strain; ○), INB201 (Δ*phoP* mutant; ▲) and M513 (Δ*afsR* mutant; ■) grown on phosphate-limited MG-3.2 (left panels) and phosphate-replete MG-18.5 (right panels). Vertical error bars correspond to the standard error of the mean of four replicated cultures.

were detected at 75 h (Fig. 1B and C), in agreement with results of Floriano and Bibb (1996).

From all available evidence, we may conclude that (i) excess of Pi inhibits Act and Red productions, being Red especially sensitive to phosphate concentrations above 10 mM; (ii) PhoP has a positive role in both antibiotics production in *S. coelicolor*, because its deletion in an otherwise isogenic strain caused a delay and reduction of Act

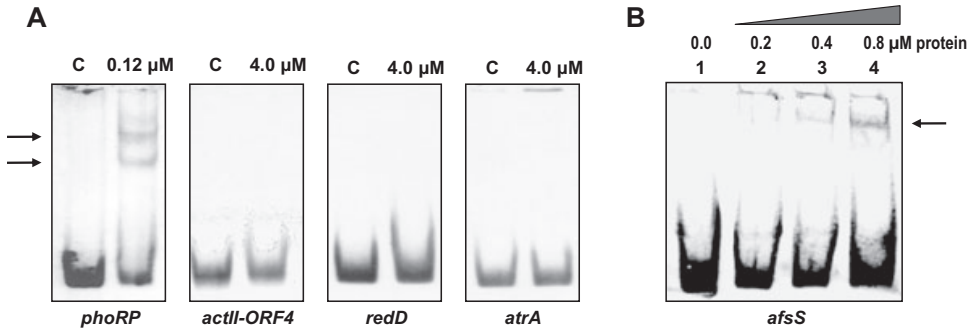

Fig. 2. A. EMSA of the *phoRP*, *act*II-ORF4, *redD* and *atrA* promoters with the GST-PhoP^{DBD} protein. The protein concentration used is indicated on top of each panel; no protein was added in the control (lane C) reaction.
B. EMSA of the *afsS* promoter with the GST-PhoP protein. Lane 1, DNA probe without protein; lane 2, 0.2 μM; lane 3, 0.4 μM, lane 4, 0.8 μM protein. Note the retarded DNA–protein complex (arrow).

and Red production; (iii) the positive effect of AfsR on antibiotic production is observed in limiting and sufficient Pi concentrations.

PhoP does not bind to the actII-*ORF4,* redD *and* atrA *promoters, but binds to the* afsS *promoter*

As an initial approach to elucidate the phosphate control of Act and Red biosynthesis, we investigated whether PhoP regulates directly the pathway-specific (cluster-located) regulators, *act*II-ORF4 and *redD*, as well as *atrA*, a transcriptional activator of *act*II-ORF4 (Narva and Feitelson, 1990; Arias *et al*., 1999; Uguru *et al*., 2005). In order to test if PhoP binds to these promoters, electrophoretic mobility shift assay (EMSA) analyses were done with the GST-PhoP^{DBD} protein, as described in *Experimental procedures*. The *phoRP* promoter known to bind PhoP (Sola-Landa *et al*., 2005) was used as positive control. Results (Fig. 2A) showed that the promoters of the three regulatory genes *act*II-ORF4, *redD* and *atrA* did not bind PhoP^{DBD}, even using 4 μM of protein, whereas the *phoRP* promoter shows retardation with 0.12 μM protein. Those results indicate that phosphate regulation of Act and Red is not exerted directly by binding of PhoP to the *act*II-ORF4 and *redD* promoters or by binding to the promoter of the *atrA* regulator.

The first characterization of PhoP operators (Sola-Landa *et al*., 2005) allowed us to detect a putative PHO box in the upstream region of the *afsS* gene by means of the 'information theory' programmes (Schneider, 1997). EMSA analyses were done to validate if this sequence is bound by PhoP. A unique retarded band was detected, even at PhoP protein concentrations as low as 0.4 μM (Fig. 2B).

Analysis of the PhoP operator in the afsS *promoter*

To determine the PhoP binding sequence in the *afsS* promoter, DNase I footprinting assays of the *afsS* promoter were carried out in the presence (0.5 μM to 1.1 μM) and absence of PhoP^{DBD} and analysed in an ALF sequencer, as described in *Experimental procedures*. These assays showed that there is a stretch of 26 nt protected both in the sense and the antisense strands of the *afsS* promoter region (Fig. 3A and B). This protected region coincides with the region reported by Lee *et al*. (2002) as the binding region of the AfsR regulator (see *Discussion*).

The PhoP binding sites are formed by two or more DRu of 11 nt.

Recently, the alignment of 37 DRu from 16 operators allowed us to build a weight matrix defined by the 'information theory' (Sola-Landa *et al*., 2008). This matrix (model I) serves to quantify the sequence conservation of new sites, given as their individual information content or R_i value (Schneider, 1997). The R_i values of model I DRu have a mean of 8.1 bits.

The PhoP-protected region in the *afsS* promoter contains two conserved DRu of R_i values 7.0 and 5.8 bits respectively (Fig. 3C). This PhoP operator belongs to class I (Sola-Landa *et al*., 2008). The two DRu are located at positions –39 to –18 with respect to the reported transcription start point of *afsS* (Lee *et al*., 2002). Thus, the –35 hexamer of the *afsS* promoter is included inside DRu-1. This same operator organization (overlapping with the -35 promoter region) is also found in the operator core of the *pitH2* gene (Santos-Beneit *et al*., 2008).

AfsR and PhoP compete for the afsS *promoter region*

To determine whether PhoP binding to the *afsS* promoter interferes or not with the binding of AfsR, both proteins were used together and separately in EMSA experiments. The H-AfsRΔC and the GST-PhoP^{DBD} proteins (both containing the DNA binding domain of the respective proteins; see *Experimental procedures*) were used. Both truncated proteins exhibited the same DNA-binding specificity as full-length proteins (Lee *et al*., 2002; Sola-Landa *et al*., 2005).

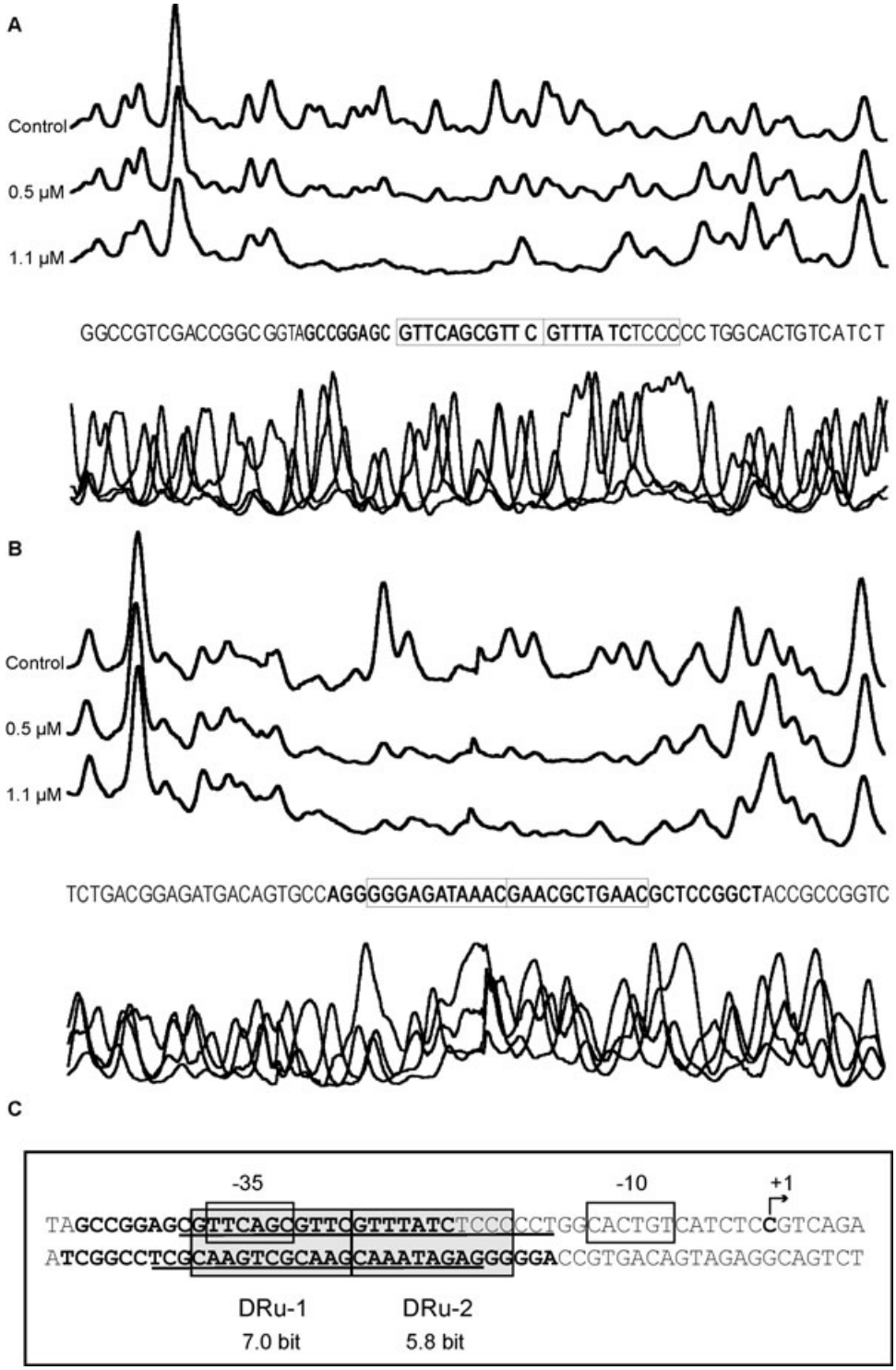

Fig. 3. DNase I footprinting of the coding (A) and the complementary (B) strands of the *afsS* promoter using GST-PhoP^{DBD}. The upper electropherograms correspond to control reaction (no protein), 0.5 μM protein and 1.1 μM protein respectively. The superimposed electropherograms correspond to sequencing reactions. The protected nucleotide sequence is indicated in boldface, and the direct repeat units (DRu) that form the PHO operator are boxed.
C. Nucleotide sequence of the *afsS* promoter. The DRu are indicated with shaded boxes; the information content of each DRu is indicated below. Sequence protected from DNase I digestion by GST-PhoP^{DBD} is in boldface; the sequence protected by AfsR (Lee *et al*., 2002) is underlined. The promoter –10 and –35 elements are boxed and the transcription start point is indicated by a bent arrow and a boldface letter (Lee *et al*., 2002).

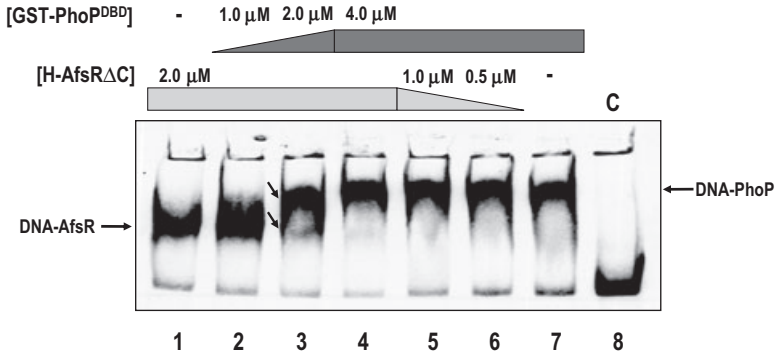

Fig. 4. Competitive EMSA of the *afsS* promoter with the H-AfsRΔC and GST-PhoP^{DBD} proteins. The end-labelled DNA fragment was incubated with the indicated concentrations of GST-PhoP^{DBD} (increasing from the left) or H-AfsRΔC (increasing from the right) proteins. Lane 8 is the control reaction with no protein added. The arrows point to the DNA–protein complexes. Note the two complexes in lane 3 (arrows). The binding buffer is that of Sola-Landa *et al.* (2005).

When applied separately, both proteins bind to the *afsS* promoter region (Fig. 4, lanes 1 and 7). In the presence of a constant H-AfsRΔC concentration, increasing the concentration of PhoP^{DBD} resulted in the disappearance of the AfsR–DNA complex and the formation of a PhoP–DNA complex (Fig. 4, lanes 1–4). Thus, PhoP can displace AfsR from the *afsS* promoter. When equimolecular protein concentrations were used the PhoP–DNA complex predominates over the AfsR–DNA complex (Fig. 4, lane 3; note the two inclined arrows). It must be noted that the binding buffer used in the experiment is that of Sola-Landa *et al.* (2005), suited for PhoP.

Transcription from the afsS *promoter is repressed by PhoP*

To investigate the role that PhoP plays on *afsS* expression, the promoter of *afsS* was coupled to the reporter luciferase gene (see *Experimental procedures*). The plasmid, pLUX-*afsS*, was introduced by conjugation in *S. coelicolor* M145 and INB201 strains and the expression of *luxAB* was followed in cultures in Pi-replete (MG-18.5) or Pi-limited (MG-3.2) media.

Luminescence data from MG-3.2 cultures revealed that the *afsS* promoter was between two and five times more active in the Δ*phoP* mutant than in the parental strain throughout the culture, except at the peak value (50 h), when both activities were similar (Fig. 5A). The expression pattern of the parental strain in MG-18.5 was similar to those of the Δ*phoP* mutant in both Pi-replete and Pi-limited cultures, except for the lower values of the mutant at 50 and 55 h in 18.5 mM (Fig. 5A). These results indicate that the phosphorylated form of PhoP controls negatively the *afsS* expression and that the lack of this regulator relieves the *afsS* promoter. This negative control may be due solely to the competition with the AfsR activator site.

As stated previously, the PHO box of *afsS* is located respect to the promoter elements in the same position as that of the *pitH2* gene, which is activated by PhoP (Santos-Beneit *et al.*, 2008). This raises the possibility that PhoP might itself act as an activator of *afsS*. To test this hypothesis, we examined the *afsS* promoter activity in a Δ*afsR* background. The pLUX-*afsS* plasmid was introduced in the Δ*afsR* mutant strain by conjugation and cultures were carried out in MG-3.2 and in MG-18.5. As expected, expression from the *afsS* promoter was impaired in the Δ*afsR* mutant, but not completely. Specific luciferase activities in Pi limitation were null at 35 and 75 h (Fig. 5B), and the maximum of 50 h was reduced to 18% of that of the wild-type peak (Fig. 5A and B). In the Pi-replete cultures, a null activity at 35 h was followed by a higher activity than in Pi-limited medium (twofold to threefold lower activities than in the wild-type values). It is interesting that the *afsS* promoter activities in the Δ*afsR* mutant were two to three times higher in Pi-replete than in the Pi-limited condition (Fig. 5B). In high Pi concentration PhoP is dephosphorylated, hence releasing its operators. Therefore, these results indicate that phosphorylated PhoP is a transcriptional repressor of *afsS*, in addition to the competitive effect with the AfsR activator.

Reciprocal regulation: AfsR also binds to the phosphate-controlled pstS *and* PhoR–PhoP *promoters*

As both global regulators PhoP and AfsR protect from DNase digestion the same sequence in the *afsS* promoter, we investigated if AfsR can bind to PHO sequences in other PhoP-regulated promoters as well. For this purpose we chose the *pstS* and *phoRP* promoter regions because they contain representative operators of class I and class III respectively (Sola-Landa *et al.*, 2008). Three versions of AfsR fusion proteins were used by Tanaka *et al.* (2007). They reported that H-AfsRΔTPR is much more stable than H-AfsRΔC or the full H-AfsR. For this reason the H-AfsRΔTPR fusion protein was used in the EMSA studies of *phoRP* and *pstS* promoters. Results of the EMSA analysis showed that *pstS* and *phoRP* promoters bind H-AfsRΔTPR even when a low (0.56 μM) concentration of protein was used. In control competition experiments, when increasing concentrations of unlabelled DNA of *pstS* or *phoRP* promoters were added, the

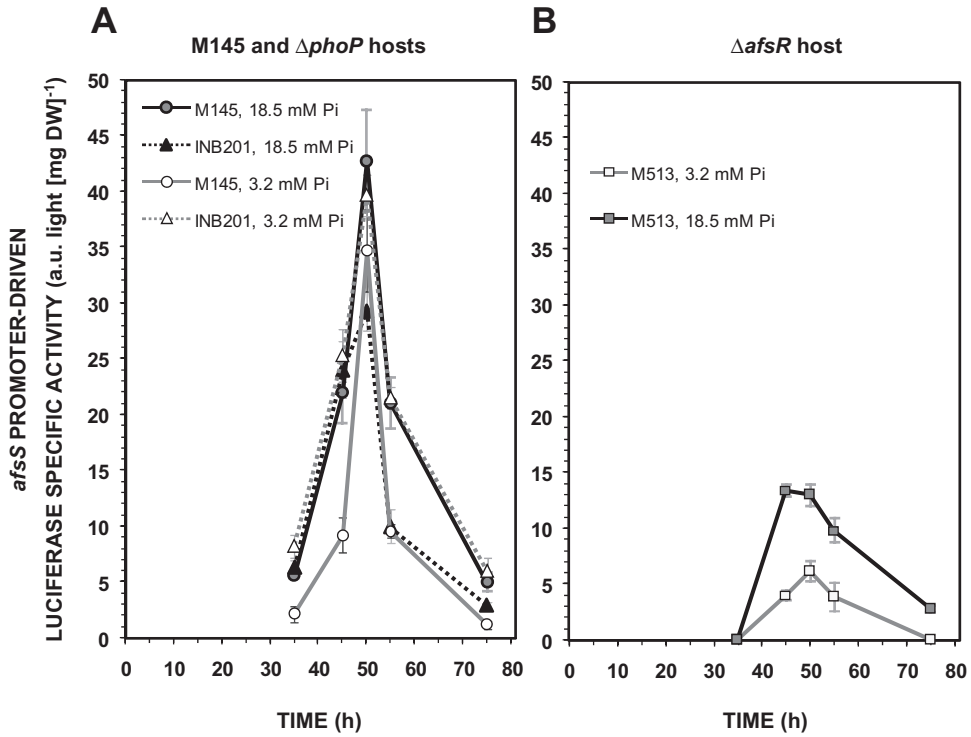

Fig. 5. Promoter activity of *S. coelicolor* exconjugants containing pLUX-*afsS* in parental strain (M145, ○) and Δ*phoP* (INB201, △) backgrounds (A), and in Δ*afsR* background (M513, □, B). The strains were grown in phosphate-limited MG-3.2 medium (grey lines and not-filled symbols), and in phosphate-replete MG-18.5 medium (black lines and filled symbols). Error bars correspond to the standard error of the mean of four culture replicates.

respective AfsR–DNA complexes were reduced in intensity (Fig. 6)., indicating that H-AfsRΔTPR binds specifically to these promoters.

Analysis of the AfsR operator sequences in the pstS *and* phoRP *promoter regions*

To determine the sequences bound by AfsR in these regions, we performed DNase I footprinting experiments using the H-AfsRΔTPR protein and a capillary sequencer to analyse the digestion fragments. As seen in Fig. 7A, the coding strand of the *pstS* promoter showed a main protected stretch of 20 nt (positions –127 to –108, respect to the translation start). To quantify the degree of protection, ratios of the areas under each fluorescence peak were calculated respect to the control reaction (i.e. a lower ratio implies a higher protection). Ratios of 81% ± 8%, 34% ± 14% and 30% ± 17% (mean ± SD) were observed at protein concentrations of 0.2, 0.8 and 1.7 μM respectively. DNase I hypersensitive positions flanked the protected sequence. H-AfsRΔTPR also bound to an upstream sequence (positions –151 to –176; Fig. 7A),

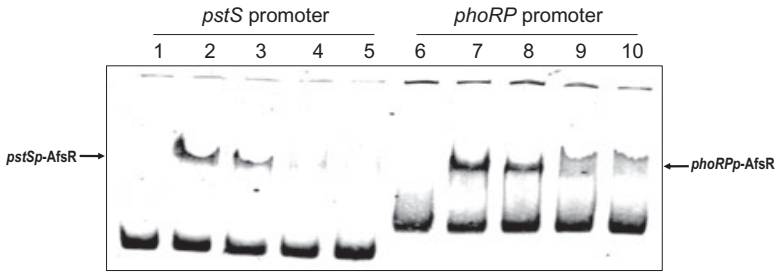

Fig. 6. EMSA of the *pstS* and *phoRP* promoters with the H-AfsRΔTPR protein and competition with unlabelled probe. Lanes 1–5, labelled *pstSp* probe; lane 1, control without protein; lane 2, 0.84 μM protein; lane 3, 0.56 μM protein; lane 4, 0.56 μM protein and 250× excess of unlabelled probe; lane 5, 0.56 μM protein and 500× excess of unlabelled probe. Lanes 6–10, labelled *phoRPp* probe; lane 6, control without protein, lane 7, 0.84 μM protein; lane 8, 0.56 μM protein; lane 9, 0.56 μM protein and 250× excess of unlabelled probe; lane 10, 0.56 μM protein and 500× excess of unlabelled probe. The arrows indicate the DNA–protein complexes.

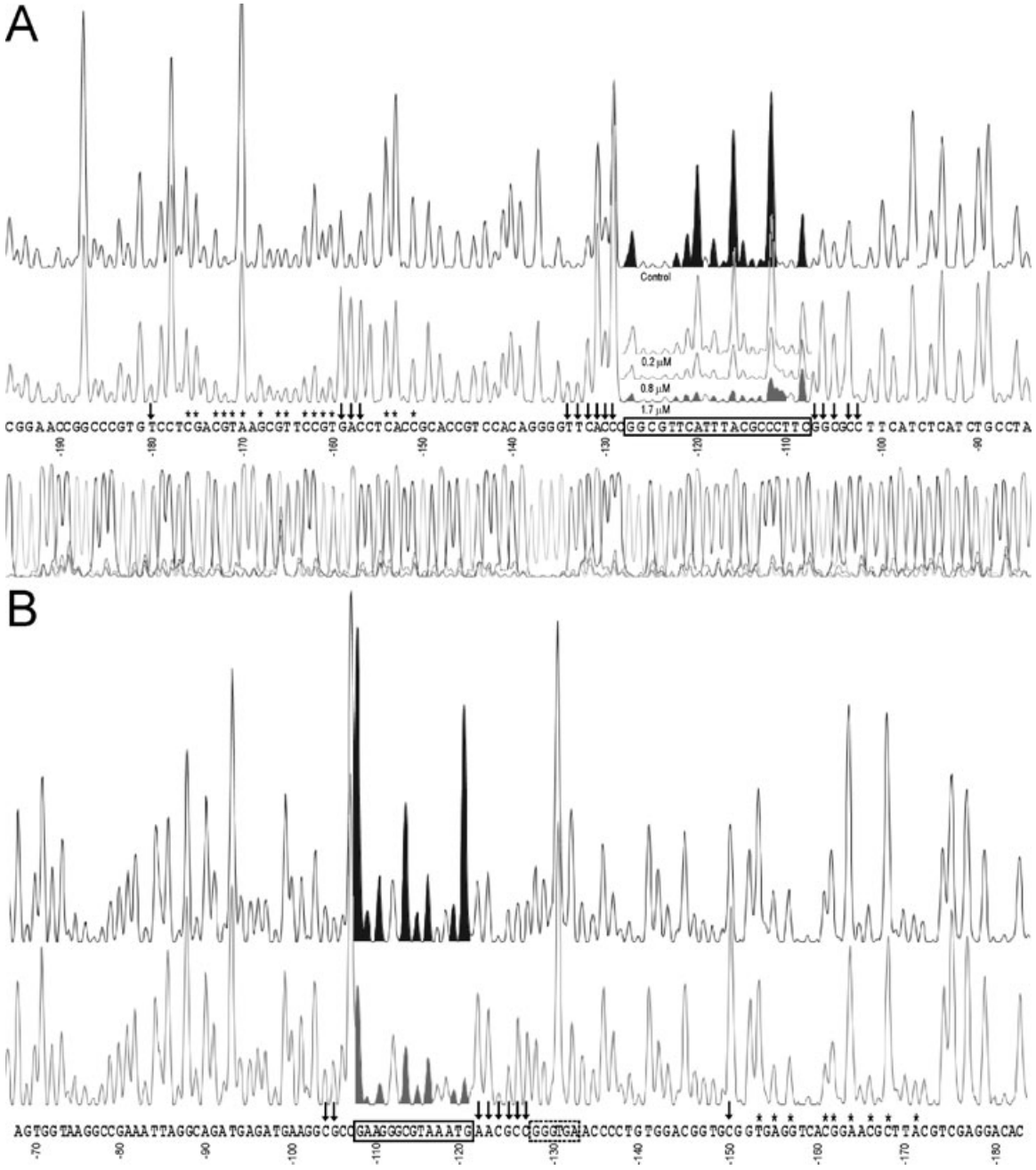

Fig. 7. DNase I footprinting of H-AfsRΔTPR binding to the *pstSp* coding (A) and complementary (B) strands, and to the *phoRPp* coding (C) and complementary strands (D). In all cases, the upper electropherogram is the control reaction without protein. In A, numbers above traces in the protected region indicate the concentrations of H-AfsRΔTPR; otherwise, 1.7 μM protein (B) and 4 μM protein (C and D) were used. The correspondence between fluorescence peaks and nucleotide bases was determined using sequencing reactions (only shown in A). Peak shadowed areas were used to calculate the degree of protection (see text). The protected sequence is boxed, or indicated by asterisks at the secondary site of *pstSp* (A and B). The vertical arrows indicate DNase I hypersensitive sites. The position respect to the translation start codon is indicated under the nucleotide sequence.

E. Summary of AfsR protection results on the *afsS* promoter (Lee *et al.*, 2002) and on the *pstS* and *phoRP* promoters. The protected sequence by AfsR in each strand is overlined or underlined. In the *pstSp*, the sequence protected to a lower extent is indicated with dotted lines. The 11 nt direct repeat units (DRu) that form the PhoP operator in *afsSp* and in *pstSp* are indicated (the sequence that contain the PHO boxes of the *phoU-phoRP* regulatory region is not included). The promoter elements (boxes) and the transcription start points (bent arrows) identified by Lee *et al.* (2002) and Sola-Landa *et al.* (2005), and the deleted nucleotide in the *pstS** mutant sequence (Δ) are also indicated.

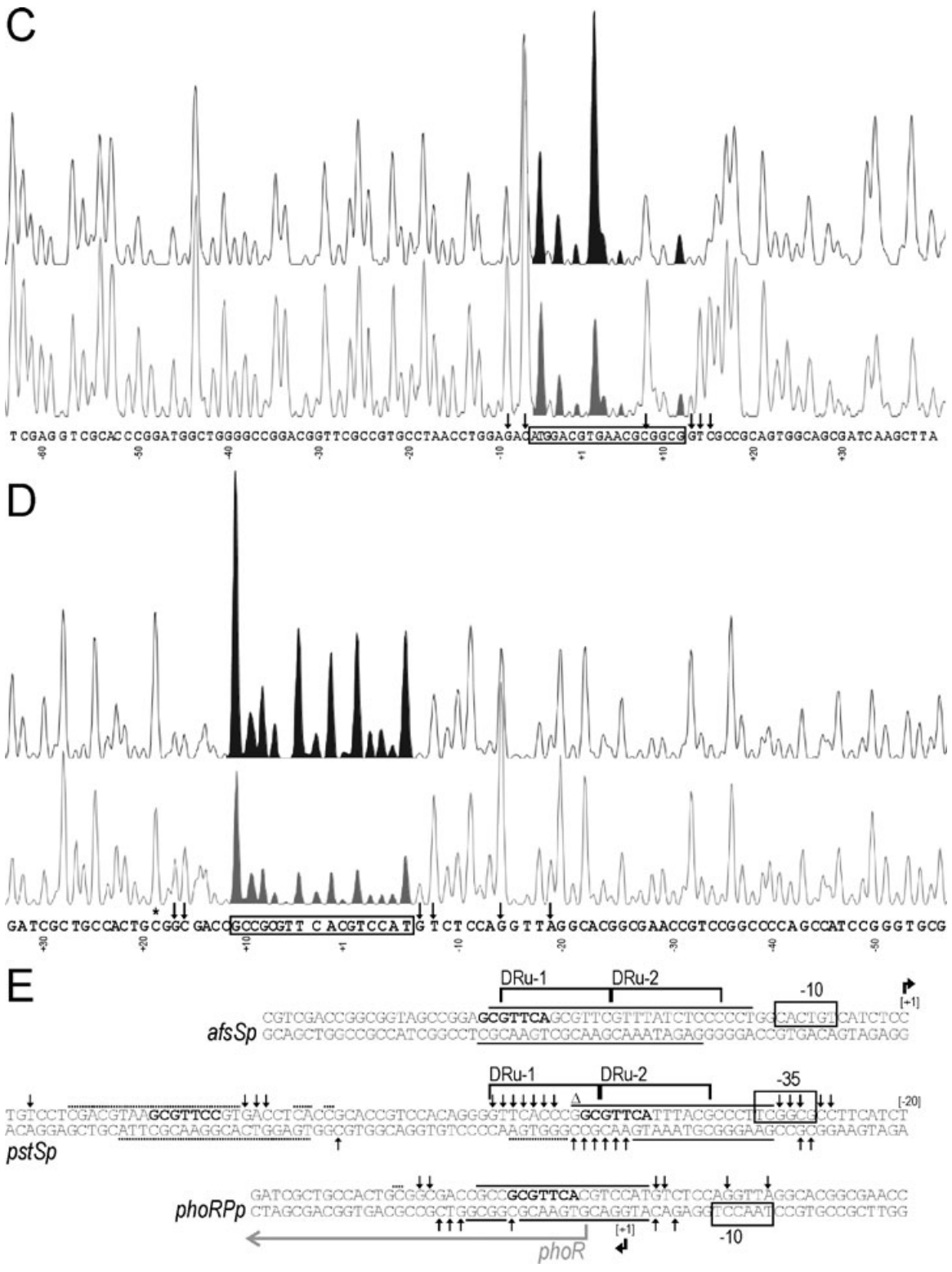

Fig. 7. *cont.*

Table 1. Luciferase specific activity (light a.u. per mg dry weight) of *pstS* promoter in M145 (parental strain) and M513 (Δ*afsR*) strains in MG-3.2.

Time	M145	M513	Ratio
40 h	$2.15 \times 10^2 \pm 0.99 \times 10^2$	$7.91 \times 10^2 \pm 1.05 \times 10^2$	3.7
44 h	$8.99 \times 10^2 \pm 0.04 \times 10^2$	$11.7 \times 10^2 \pm 1.96 \times 10^2$	1.3
48 h	$13.2 \times 10^2 \pm 1.47 \times 10^2$	$20.1 \times 10^2 \pm 1.08 \times 10^2$	1.5
52 h	$2.78 \times 10^2 \pm 0.15 \times 10^2$	$2.30 \times 10^2 \pm 0.50 \times 10^2$	0.8
64 h	$0.45 \times 10^2 \pm 0.20 \times 10^2$	$1.28 \times 10^2 \pm 0.22 \times 10^2$	2.9

Mean and SD of two independent cultures are shown.

but with lower affinity, because area fractions were 95% ± 5%, 76% ± 7% and 58% ± 15% of that of the control at the above-indicated protein concentrations respectively.

Footprinting of the complementary strand confirmed the specific binding to both sites (Fig. 7B). Peak ratios of the main binding site (positions from –121 to –108), were 76% ± 7%, 42% ± 14% and 40% ± 23% at the above protein concentrations. DNase I hypersensitive sites surrounded this region. The adjacent hexamer (GGGTGA) appeared protected to a lower extent (peak ratios of 100% ± 4%, i.e. null protection; 91% ± 9% and 64% ± 17%, respectively, to the indicated protein range). Protection of the secondary binding site (positions –153 to –171) in the complementary strand was reflected by the peak ratios 99% ± 2%, 92% ± 5% and 79% ± 7% respectively.

The *phoRP* promoter was protected by H-AfsRΔTPR at a unique region located at positions –6 to +12 in the coding strand (Fig. 7C), and –6 to +11 in the non-coding strand (Fig. 7D). Also DNase I hypersensitive sites were present in the flanking positions. The protein affinity to this sequence was lower than the affinity to the main *pstSp* binding site because a higher protein concentration was required to obtain similar protection. Thus, 4 μM protein yielded peak ratios of 63% ± 19% in the coding strand (Fig. 7C), and of 37% ± 15% in the non-coding strand (Fig. 7D).

The summary of these results is shown in Fig. 7E. As indicated previously, the binding site of the AfsR regulator overlapped completely the PHO box in the *afsS* promoter. In the *pstS* promoter the AfsR binding site only covered the last DRu of the PhoP operator, which comprises two DRu, and in the *phoRP* promoter the AfsR site is completely separated from the PhoP operator.

Previously, we reported that deletion of the 10th bp of the first DRu disrupted the binding of PhoP to the *pstS* operator (Sola-Landa *et al.*, 2008; Fig. 7E). We used this mutant sequence (*pstS**) to check if the first DRu participates in the AfsR binding. The H-AfsRΔTPR protein retarded and protected similarly this *pstS** fragment as well as the wild-type sequence (data not shown). As the only difference was that the protection of the *pstS** coding strand lacked the deleted nucleotide (starting GCGT TCA . . . ; Fig. 7E), we may conclude that the first DRu of the PhoP is not involved in the AfsR binding. In summary, PhoP and AfsR recognize overlapping (or adjacent) but different nucleotide sequences.

AfsR represses the transcription of the pstS *gene*

The plasmid pLUX-*pstS* was used previously as a reporter of the *pstS* promoter activity in Pi shift-down experiments, because expression of this gene is strongly dependent upon PhoP and serves to monitor the PHO response *in vivo* (Rodríguez-García *et al.*, 2007). To determine the sign of the AfsR regulation on this gene, this plasmid was introduced into the M145 and M513 (Δ*afsR*) strains. Cultures were done only in MG-3.2 medium, because the *pstS* promoter is not activated in high Pi concentrations (Santos-Beneit *et al.*, 2008). The lack of AfsR caused consistently higher *pstS* promoter activities through most of the time-course of the experiment (Table 1). This negative effect of AfsR on the expression of *pstS* might explain why under Pi-limited conditions the growth of the Δ*afsR* strain was higher than the parental strain (Fig. 1).

Discussion

The two-component system PhoR–PhoP controls the response to Pi scarcity in *Streptomyces* species (Sola-Landa *et al.*, 2003; 2005; Ghorbel *et al.*, 2006). In this work we focused on the search of PhoP target sequences (PHO boxes) in the model organism *S. coelicolor* to explain the role of the PhoR–PhoP system in secondary metabolism. As a first approach, we cloned three promoters of antibiotic regulatory genes. PhoP failed to bind the promoters of *act*II-ORF4, *redD* and *atrA*, thus excluding a direct regulation of these pathway-specific and AtrA transcriptional regulators.

Bioinformatic searches allowed the identification of a putative PHO box in the *afsS* promoter that overlaps with the AfsR binding sequence. We concluded from gel-retarding and footprinting assays *in vitro*, and promoter–

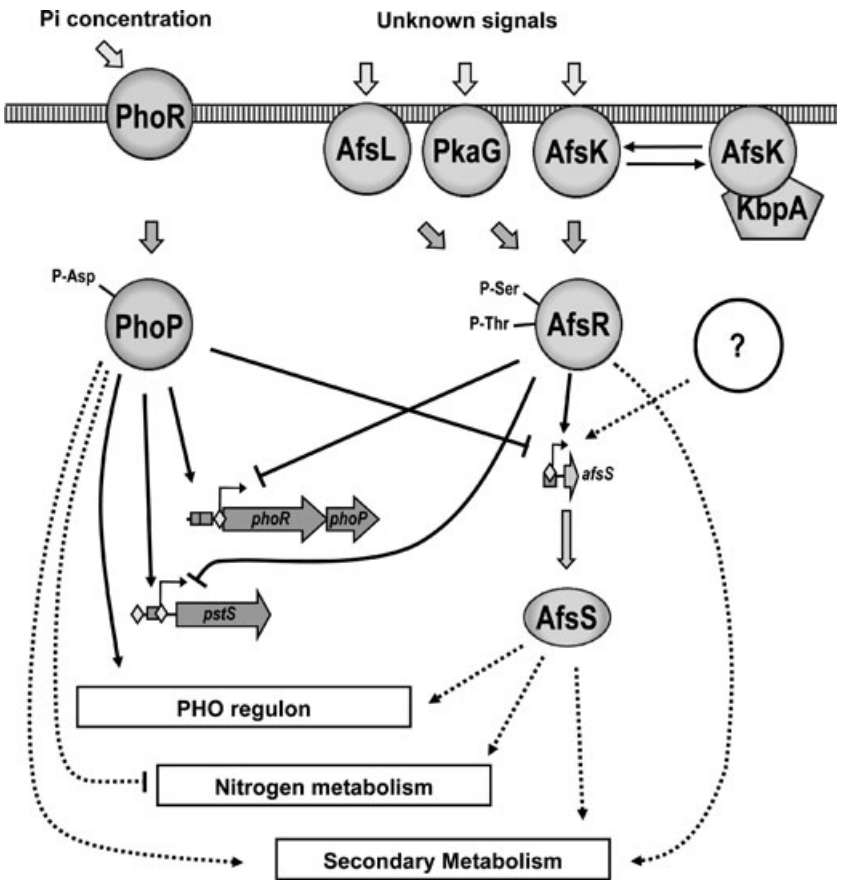

Fig. 8. Model of signal transduction and cross-talk regulation in PhoR–PhoP and AfsK–AfsR–AfsS systems. The model takes into account the data of Horinouchi (2003) and the results of Sola-Landa *et al.* (2005), Rodríguez-García *et al.* (2007), Lian *et al.* (2008) and this work. PHO boxes are represented by a small square and AfsR binding sites by a diamond.

luciferase genes fusions *in vivo*, that PhoP represses the transcription of *afsS*. Two mechanisms account for this repression: first, PhoP is an anti-activator (Browning and Busby, 2004) because it competes with AfsR; second, PhoP blocks the RNA polymerase binding to the promoter by steric hindrance. To our knowledge, this is the first description of a complete signal transduction cascade from a nutrient-sensing (Pi) to an antibiotic regulator (Fig. 8).

Very little is known about the signal transduction pathways that take place upstream of the specific antibiotic regulators. In bacteria, signal transduction is mediated by protein phosphorylation of two-component systems, and by serine/theonine protein kinases. One of the first regulatory cascades discovered in bacteria that involve serine/theonine kinases was indeed the AfsK–AfsR–AfsS system (Horinouchi, 2003). AfsK is a loosely attached membrane kinase that first phosphorylates itself and then the serine and threonine residues of the pleiotropic regulator AfsR (Matsumoto *et al.*, 1994). Another protein, KbpA, inhibits the autophosphorylation of AfsK (Umeyama and Horinouchi, 2001). Other two serine/threonine kinases, AfsL and PkaG, were also found to phosphorylate AfsR (Sawai *et al.*, 2004), which suggests that AfsR integrates multiple inputs. It is not yet known what kind of environmental signals trigger the system, although AfsK appears to bind S-adenosyl-L-methionine (Lee *et al.*, 2007). Phosphorylated AfsR binds the –35 region of the *afsS* promoter and activates its transcription (Lee *et al.*, 2002; Tanaka *et al.*, 2007). When introduced in a high-copy-number plasmid, *afsS* enhances the production of Act and Red in both *S. lividans* and *S. coelicolor* (Vögtli *et al.*, 1994; Matsumoto *et al.*, 1995; Floriano and Bibb, 1996). Although *afsS* stimulates the expression of the pathway-specific transcriptional regulators *act*II-ORF4 and *redD* respectively, the molecular mechanism of this activation is unknown (Horinouchi, 2003). In addition, the results of Floriano and Bibb (1996) and Lee *et al.* (2002) indicated that the positive effect of AfsR in secondary metabolism involves genes other than *afsS*. Indeed, we report herein the existence of three new AfsR binding sites in the promoters of key phosphate transport and regulatory genes.

DNase I footprinting assays showed that AfsR protects two separate sequences in the *pstS* promoter. The higher-affinity site overlaps partially with the –35 element (Fig. 7E), but does not meet the strict requirements to be an activator site (Tanaka *et al.*, 2007). Thus, when AfsR is bound at this sequence, it should prevent the binding of both the PhoP and the RNA polymerase. The lower-

affinity site is placed more than 40 nt upstream of the –35 element. Distal sites may act as repressors when the protein binding induces a loop in the DNA (Browning and Busby, 2004), and it is possible that this secondary site reinforces *pstS* repression at higher AfsR concentrations. Besides, luciferase reporter assays confirmed that AfsR represses the *pstS* gene.

The third new AfsR binding site was found at the *phoRP* transcription start (Fig. 7E). The position and the inverse orientation of the binding-site, compared with that of the *afsS* activator, strongly indicate that AfsR blocks the RNA polymerase. Repressors that bind downstream of the transcriptional start point are common (Babu and Teichmann, 2003). Blocking the transcription at this site is expected to affect the expression of the two components PhoR–PhoP of the phosphate-responsive system that appear to be cotranscribed in *S. coelicolor*.

Although both AfsR and PhoP proteins bind to the same region in the *afsS* promoter, they must have distinct sequence specifities, because AfsR protects sequences that PhoP does not bind (e.g. the site upstream of the *pstS* PHO box, the site that overlaps the start codon of *phoR*, and the mutant sequence *pstS**) and because AfsR does not protect the PHO boxes in the *phoU-phoRP* intergenic region (Fig. 7).

Tanaka *et al*. (2007) indicated that the AfsR binding site of the *afsS* promoter contains two direct repeats (consensus sequence CGTT(T/C)ATCGNN). However, there is no apparent conservation of a second repeat in the other three operators located in the *pstS* and *phoR* genes (Fig. 7E). This suggests that the clear sequence repetition found in the *afsS* promoter is due mostly to the PhoP binding site and not to the AfsR recognition sequence. Alignment of the four known AfsR binding sequences only revealed conservation of the sequence GCGTTC(A/C). Clarification of the sequence pattern recognized by AfsR will require detection of additional operators and directed mutagenesis studies.

As shown in flask liquid cultures of wild-type and mutant strains in defined MG medium, PhoP has an overall positive role in *S. coelicolor* Red and Act production. As PhoP represses *afsS*, which positively controls Red and Act production, it should influence other mechanisms to account for the overall positive effect.

This positive effect of PhoP contrasts with previous results of our group in *S. lividans* and in *Streptomyces natalensis* liquid cultures using complex media (Sola-Landa *et al*., 2003; Mendes *et al*., 2007). Disruptions of *phoRP* or *phoP* alone lead to Act and Red synthesis in R5 cultures of *S. lividans* (Sola-Landa *et al*., 2003). The pimaricin production of the *S. natalensis* Δ*phoRP* strain was higher than that of the wild-type strain in NBG medium (composed of NaCl, beef extract and glucose; Mendes *et al*., 2007). Indeed the regulation of antibiotic production in *S. lividans* is different from that of *S. coelicolor*, because *S. lividans* usually does not synthesize Act or Red in common laboratory media. Differences between *S. lividans* and *S. coelicolor* have been reported on glucose repression of Act (Kim *et al*., 2001), and on the stimulatory effect of multiple copies of *afsS* in *afsR* mutant backgrounds (Floriano and Bibb, 1996). Thus, the regulatory features of PhoP might vary across species producing different antibiotics.

As expected from the results of other laboratories, the absence of AfsR in the M513 mutant led to a lower antibiotic production. Our results add new information on the AfsR–AfsS regulation. Thus, the *afsSp*-driven specific luminescence is reduced, but not abolished, in the *afsR* mutant grown on liquid MG medium. This indicates that other regulatory factors may also activate the *afsS* promoter, such as one of the three AfsR-like proteins encoded in the *S. coelicolor* genome (Bentley *et al*., 2002).

The cross-regulation of PhoR–PhoP and AfsK–AfsR–AfsS systems that is described here at the molecular level complements the recent work of Lian *et al*. (2008). These authors compared the transcriptomic profiles of wild-type and *afsS* mutant strains in liquid cultures, and found that AfsS positively controls some PHO regulon genes, including the *phoRP* system itself. Thus, AfsS produces the opposite effect on the expression of the PHO regulon genes than AfsR. This intricate cross-regulation is summarized in Fig. 8. Growing evidence indicates that the main regulatory systems in the soil-dwelling bacteria *Streptomyces* are intertwined. In addition to Pi, the carbon source modulates the effect of AfsR and AfsS (Floriano and Bibb, 1996; Sekurova *et al*., 1999; Kim *et al*., 2001), as well as the expression of *pstS* (Díaz *et al*., 2005). Key genes of the nitrogen metabolism are upregulated in the *phoP* mutant strain (Rodríguez-García *et al*., 2007), but are downregulated in the *afsS* mutant strain (Lian *et al*., 2008). In summary, *Streptomyces* have developed intertwined mechanisms to adapt their metabolism to the changing nutritional conditions in the soil.

Experimental procedures

Bacterial strains, plasmids and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 2. As this work is part of the European systems biology project of *S. coelicolor*, we used the strain provided by Eriko Takano as the parental strain. This is a single colony isolate of the widely used M145 strain (Kieser *et al*., 2000). The *S. coelicolor* INB201 Δ*phoP* mutant was obtained for this work by the replacement procedure already used in Rodríguez-García *et al*. (2007), except that the parental of INB201 was the above mentioned strain. *S. coelicolor* M513, an in-frame deletion mutant of the *afsR* gene (Floriano and Bibb, 1996), was provided by Mervin Bibb. All strains were

Table 2. Bacterial strains and plasmids used in this work.

Strains/plasmids	Characteristics	Reference
Strains		
Streptomyces coelicolor A3(2) M145	Plasmid-free derivative of the Wild-type *S. coelicolor* A3(2) strain	Kieser *et al*. (2000)
Streptomyces coelicolor A3(2) INB201	Δ*phoP,* Am^r	This work
Streptomyces coelicolor A3(2) M513	Δ*afsR*	Floriano and Bibb (1996)
Escherichia coli DH5α	F′Φ80 *dLacZ* ΔM15	Hanahan (1983)
Escherichia coli ET12567 (pUZ8002)	*dam dcm* mutant, Neo^r -Cm^r	MacNeil *et al*. (1992)
Escherichia coli BL21 (DE3) pLysS	F$^-$ *ompT hsdS$_B$* (r_{B^-} m_{B^-}) *gal dcm* (DE3)pLysS (cam^R)	Novagen
Plasmids		
pGEM-T-easy	Cloning vector, Amp^r	Promega
pGEM-P*redD*	PCR product carrying *redD* promoter cloned into pGEM-T-easy, Amp^r	This work
pGEM-P*actIIORF4*	PCR product carrying *actIIORF4* promoter cloned into pGEM-T-easy, Amp^r	This work
pGEM-P*atrA*	PCR product carrying *atrA* promoter cloned into pGEM-T-easy, Amp^r	This work
pBS-P*phoU*	PvuI fragment carrying *phoU* promoter cloned into pBluescript KS+, Amp^r	Sola-Landa *et al*. (2005)
pGEM-P*pstS*	PCR product carrying *pstS* promoter cloned into pGEM-T-easy, Amp^r	Sola-Landa *et al*. (2005)
pGEM-P*pstS**	PCR product carrying *pstS** promoter cloned into pGEM-T-easy, Amp^r	Sola-Landa *et al*. (2008)
pBluescript II SK+	Cloning vector, Amp^r	Stratagene
pFS-*afsS*	PCR product carrying *afsS* promoter cloned into pBSIISK+, Amp^r	This work
pLUXAR-neo	Integrative promoter-probe vector, *luxAB* genes, Am^r, Neo^r	This work
pLUX-*afsS*	BamHI-NdeI pFS-*afsS* fragment cloned into pLUXAR-neo, Am^r, Neo^r	This work
pLUX-*pstS*	BamHI-NdeI pGEM-P*pstS* fragment cloned into pLUXAR+, Am^r	Santos-Beneit *et al*. (2008)
pET16-*afsR*ΔC	Cloning vector carrying His-tagged truncated AfsR (Met1 to Ala270), Amp^r	Lee *et al*. (2002)
pET16-*afsR*ΔTPR	Cloning vector carrying His-tagged truncated AfsR (Met1 to Glu618), Amp^r	Tanaka *et al*. (2007)
pGEX-PhoP	*S. coelicolor phoP* gene cloned into pGEX-2T	Sola-Landa *et al*. (2005)
pGEX-DBD	*S. coelicolor phoP*-DBD gene cloned into pGEX-2T	Sola-Landa *et al*. (2005)

manipulated and conjugated according to standard procedures (Kieser *et al*., 2000). *Escherichia coli* DH5α was the general cloning host.

The *afsS* promoter region was amplified by PCR using total DNA as template, as follows: the primers CAR21 (5′-ATGCGGATCCTCAGCCTCTACGAGCAGC) and CAR22 (5′-TGGGTACCATATGTCCGCGTCCTTCATCTTG) amplified a 339 bp fragment encompassing the intergenic region between *afsR* and *afsS*, the last 77 bp of *afsR* and the first 26 bp of *afsS*. The BamHI (CAR21) and KpnI/NdeI (CAR22) cloning sites were introduced in the primer sequences. A BamHI-KpnI fragment was cloned into pBluescript II SK+ obtaining pFS-*afsS*. The insert of the plasmid was checked by sequencing. A BamHI-NdeI fragment of 321 bp from pFS-*afsS* was cloned into the promoter-probe vector pLUXAR-neo, yielding pLUX-*afsS*. pLUXAR-neo was constructed from pLUXAR+ (Rodríguez-García *et al*., 2007) by insertion of the *neo* gene at the NheI site, downstream of the *aac(3)IV* gene.

redD, *act*II-ORF4 and *atrA* promoters were amplified by PCR using the primer pairs *redD*-Up (5′-CGCGGGATCC GATGATGTTGGAGTTG) and *redD*-Low (5′-CCCCTCTAG ACATATGCCGAACGATCGGATTC), *act*II-Up (5′-GGACGG ATCCGAGGACCCAGCCGTATCAG) and *act*II-Low (5′-AG TTTCTAGACATATGCGCCCCCGTC), and *atrA*-Up (GGATC-CGCTCGTCAAATTGTGTGACCGG) and *atrA*-Low (5′-TCTAGAATAGACAATCCCCCGGTAATGACG) respectively. Final products of PCR (533 bp, 241 bp and 353 bp respectively) were cloned into pGEM-T-easy (Promega) and the correct amplification was confirmed by sequencing.

Streptomyces coelicolor cultures were performed in defined MG medium containing starch (Scharlau; 50 g l^{-1}) and glutamate (60 mM; Doull and Vining, 1989). One hundred millilitres of MG medium in 500-ml baffled flasks were inoculated with 10^6 spores ml^{-1} and incubated at 30°C, 300 r.p.m. (25.4 mm orbit diameter) for reproducible and dispersed growth. For the phosphate-replete and the phosphate-limited conditions cultures contained 18.5 mM and 3.2 mM potassium phosphate respectively (MG-18.5 and MG-3.2 mediums; Santos-Beneit *et al*., 2008). Samples to measure *afsSp* activity, antibiotic production and growth were taken at 35 h, 45 h, 50 h, 55 h and 75 h of incubation. In all cases two different exconjugants were selected for the cultures. Duplicates of each exconjungant were used (four replicates of each condition). For *pstSp–luxAB* reporter studies samples were taken at 40 h, 44 h, 48 h, 52 h and 64 h of incubation. In this case, only one exconjugant was used for each strain. Both cultures were done in duplicate.

Luciferase assay, growth and antibiotic production determinations

The *luxAB* activity driven from the *afsS* promoter was determined in a Luminoskan luminometer (Labsystems, Helsinki) as reported in Rodríguez-García *et al*. (2007) and Santos-Beneit *et al*. (2008). Cell pellets from 1 ml culture samples were kept frozen until luminescence readings. The *pstSp–luxAB* gene fusion activity was measured as stated

above, but the samples were kept on ice and measured immediately.

Antibiotic assays were performed as described by Kieser *et al*. (2000). For dry weight determination, culture samples of 2 ml were washed twice with MilliQ water and dried during 4 days at 80°C.

Production and purification of H-AfsRΔC, H-AfsRΔTPR, GST-PhoP and GST-PhoP^{DBD} proteins

H-AfsRΔC (291 amino acids, 32 kDa; containing the N-terminal portion of AfsR from Met-1 to Ala-270; Lee *et al.,* 2002) and H-AfsRΔTPR (639 amino acids, 68 kDa; containing the N-terminal and central portions of AfsR from Met-1 to Glu-618; Tanaka *et al*., 2007) histidine-tagged proteins were purified from the soluble fractions of *E. coli* BL21 (DE3) pLysS harbouring the plasmids pET16-*afsR*ΔC and pET16-*afsR*ΔTPR respectively, as described by Lee *et al*. (2002). The purified samples were dialysed overnight against 10 mM Tris-HCl (pH 7) and 10% glycerol.

GST-PhoP (449 amino acids, 51 kDa; containing a full-length PhoP construct fused to the GST protein) and GST-PhoP^{DBD} (341 amino acids, 39 kDa; containing the C-terminal portion of PhoP from Ile-109 to Pro-223; Sola-Landa *et al*., 2005) proteins were purified from the soluble fractions of *E. coli* DH5α harbouring the plasmids pGEX-PhoP and pGEX-DBD respectively, as described by Sola-Landa *et al*. (2005).

Protein concentration was determined with the Bradford reagent (Bio-Rad).

Gel mobility shift assays

The promoters were excised from its respective plasmids by digestion with restriction endonucleases and labelled at both ends with digoxigenin using the DIG Oligonucleotide 30-End Labeling Kit, 2nd Generation (Roche Applied Science). The sizes of the DNA probes were the following: 321 bp (*afsS*), 315 bp (*phoRP*), 280 bp (*pstS*), 279 bp (*pstS**), 553 bp (*redD*), 261 bp (*act*II-ORF4) and 373 bp (*atrA*). The conditions for DNA–protein binding and detection were described previously by Sola-Landa *et al*. (2005). As the H-AfsRΔC solution differs from the GST-PhoP^{DBD} one, the binding reactions in the competitive EMSA were equalized adding different amounts of SAD (10% glycerol and 10 mM Tris-HCl pH 7.0) and SPD (40% glycerol, 25 mM Tris-HCl pH 8.0 and 5 mM reduced glutathione).

Samples were run in 0.5× TBE buffer on a 5% polyacrylamide native gel during 2 h at 80 V using a Bio-Rad Mini Protean III apparatus.

DNase I footprinting

The DNase I footprinting assays of *afsS* promoter were performed using the GST-PhoP^{DBD} protein as described in Sola-Landa *et al*. (2005). DNA probes for both the coding and complementary strands were obtained by PCR using pFS-*afsS* as template and the respective fluorescent-labelled primer. The labelled probes (495 bp and 490 bp for the coding and complementary strand respectively) were purified from agarose electrophoresis (GFX columns). DNase I footprinting was performed by incubating 0.28 pmol of the DNA probe with different concentrations of the GST-PhoP^{DBD} protein during 30 min at 30°C. DNase I digestions were carried out during 1 min at 30°C. Thermo Sequenase Primer Cycle Sequencing Kit (GE Healthcare) and labelled primers were used to obtain reaction sequences. The reaction products were resolved in an ALF DNA sequencer (GE Healthcare) and analysed with the Fragment Manager program as described previously (Rodríguez-García *et al*., 1997).

The DNase I footprinting assays of *phoRP, pstS* and *pstS** promoters with the H-AfsRΔTPR protein were carried out as above, but using the SAD buffer instead of SPD. For these footprinting assays we used the 6-FAM-labelled primers CAGGAAACAGCTATGAC (reverse) and CGACGTTGTAAAACGACGGCCAGT (forward). Labelled PCR products were added to a final concentration of 9.3 nM. Each reaction was loaded into an ABI PRISM 3130 sequencer together with the molecular standard Gene-Scan® 500 LIZ™ (Applied Biosystems). Electrophoregrams were analysed with Peak Scanner software v1.0 (Applied Biosystems) and normalized against the control. For this purpose, the total peak area was calculated for the regions shown in Fig. 7, excluding protected peaks.

'Information theory' analysis of binding sites

To evaluate the potential binding of PhoP, we calculated the individual information content (Schneider, 1997) of each 11 nt stretch using the weight matrix of model I of Sola-Landa *et al*. (2008). This weight matrix gathers the information of 37 DRu that form the core of experimentally demonstrated PhoP operators.

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