Molecular Microbiology (2005) **56**(5), 1373–1385 doi:10.1111/j.1365-2958.2005.04631.x

Binding of PhoP to promoters of phosphate-regulated genes in *Streptomyces coelicolor*: identification of PHO boxes

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Summary

The control of phosphate-regulated genes in *Streptomyces coelicolor* is mediated by the two-component system PhoR–PhoP. When coupled to the reporter *xylE* gene the *pstS*, *phoRP* and *phoU* promoters were shown to be very sensitive to phosphate regulation. The transcription start points of the *pstS*, the *phoRP* and the *phoU* promoters were identified by primer extension. *phoRP* showed a leaderless transcript. The response-regulator (DNA-binding) PhoP protein was overexpressed and purified in *Escherichia coli* as a GST–PhoP fused protein. The DNA-binding domain (DBD) of PhoP was also obtained in a similar manner. Both PhoP and its truncated DBD domain were found to bind with high affinity to an upstream region of the *pstS* and *phoRP–phoU* promoters close to the –35 sequence of each of these promoters. DNase I protection studies revealed a 29 bp protected stretch in the sense strand of the *pstS* promoter that includes two 11 bp direct repeat units. Footprinting of the bidirectional *phoRP–phoU* promoter region showed a 51 bp protected sequence that encompasses four direct repeat units, two of them with high similarity to the protected sequences in the *pstS* promoter. PHO boxes have been identified by alignment of the six direct repeat units found in those promoter regions. Each direct repeat unit adjusts to the consensus $G^{G/T}TCAYYYR^{G/C}G$.

Introduction

Over the last four decades an impressive number of antibiotics and other secondary metabolites have been shown to be regulated negatively by phosphate (Masuma *et al*., 1986; Martín, 1989; Liras *et al*., 1990) but, surprisingly, the molecular mechanism of phosphate control has remained obscure (Martín and Demain, 1980; Martín *et al*., 1994) in spite of its basic and industrial relevance. It is interesting to note that inorganic phosphate in the culture media controls the synthesis of a large number of secondary metabolites belonging to different biosynthetic groups, e.g. macrolides, tetracyclines, anthracyclines, polyether compounds, aminoglycosides, amino acid-derived metabolites such as clavulanic acid, among others (Dekeva *et al*., 1985; Doull and Vining, 1990; Hobbs *et al*., 1990; 1992; Lounes *et al*., 1996). From a biosynthetic point of view, these groups of metabolites have very little in common, except that they all are dispensable 'secondary' metabolites (Vining, 1992; Martín *et al*., 2000). Martín and Demain (1980) proposed that phosphate control is used as a mechanism that triggers secondary metabolite biosynthesis when phosphate in the environment is depleted and therefore growth of the microorganisms cannot proceed at a normal rate (Demain and Vaishnav, 2004). When phosphate concentration in the culture media decreases below a threshold level, bacteria increase their production of a variety of metabolites that might serve as direct antagonists to other microorganisms (Vögtli *et al*., 1994) or as biochemical cross-talk signals (Horinouchi and Beppu, 1992; Vining, 1992; Kaiser and Losick, 1993) to enhance survival under harsh nutritional conditions (Mapplestone *et al*., 1992).

Recently, we reported that phosphate control of antibiotic biosynthesis in *Streptomyces lividans* and *Streptomyces coelicolor* is mediated by the two-component PhoR–PhoP system (Sola-Landa *et al*., 2003) that also controls the alkaline phosphatase gene (*phoA*) and other *phoA*-related genes (K. Apel, A. Sola-Landa, A. Rodríguez-García and J.F. Martín, unpublished). The *S. coelicolor* and *S. lividans* PhoRP systems belong to class IIIA of two component systems (Fabret *et al*., 1999; Hutchings *et al*., 2004). The PhoR protein is a standard membrane sensor kinase, and PhoP is a member of the OmpR family of DNA binding response regulators. In *Escherichia coli* and *Bacillus subtilis*, the phosphorylated PhoP protein (PhoP~P) in response to phosphate starvation activates expression of the *pho* regulon genes by binding to PHO boxes in the promoter regions (Torriani-Gorini, 1994; Hulett, 1996). In

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S. lividans expression of *phoA* is induced by PhoP~P and mutants lacking *phoP* (or the *phoR–phoP* cluster) do not form PhoA. These mutants overproduce large amounts of actinorhodin and undecylprodigiosin (Sola-Landa *et al*., 2003).

PhoP, as other members of the response regulator OmpR family, contains the DNA-binding domain (DBD) in the C-terminal region (amino acids 187–201) (Sola-Landa *et al*., 2003). In *E. coli* it is known that when the N-terminal region of PhoB (homologous to the *Streptomyces* PhoP) is deleted, including part or all of the α5 helix, the truncated protein binds constitutively to the PHO boxes with higher affinity than the unmodified protein (Ellison and McCleary, 2000; Allen *et al*., 2001). It was therefore of interest to investigate whether the C-terminal *S. coelicolor* PhoP domain (hereafter named $PhoP^{DBD}$) was able to recognize the putative PHO boxes with higher or decreased affinity than intact PhoP. We report in this article the binding of $PhoP^{DBD}$ to upstream sequences of three different phosphate-regulated *S. coelicolor* promoters. Footprinting analysis has allowed the identification of so far unknown PHO boxes in the promoter of genes of the *pho* regulon in *S. coelicolor*.

Results

Expression from phoRP*,* phoU *and* pstS *promoters is strongly regulated by inorganic phosphate*

The two promoters of the intergenic region between *phoRP* (encoding the sensor kinase and the response regulator) and *phoU* (a modulator of the phosphate response) genes (Fig. 1) were selected for this study because they are likely to be regulated by the PhoP protein.

A third promoter corresponding to the *pstS* (phosphate specific transfer) gene was also studied because in preliminary studies of phosphate-regulated promoters it was found to be very sensitive to phosphate control. The *pstS* is the first gene of a cluster involved in high-affinity phosphate uptake in media containing low phosphate concentration (Torriani-Gorini, 1994). The encoded PstS is a phosphate-binding protein. Upstream of the *pstS* gene and in the same orientation there are two genes homologous to the *mutT* and to the diadenosine 5′, 5‴-P_1, P_4-tetraphosphate pyrophosphohydrolase genes (Bentley *et al*., 2002), respectively, and downstream there are other *pst* genes (Fig. 1).

Using the *xylE* gene (encoding catechol 2,3-dioxygenase) as reporter, the regulatory effect of phosphate on expression of these three genes was studied in *S. coelicolor* liquid cultures in R5 medium using high (1.85 mM) or low (40 μM) phosphate concentrations. Results (Fig. 2) showed that the three promoters are strongly repressed (about 90% reduction in expression) by high phosphate concentrations.

Both *phoR* and *phoU* genes that are expressed from the bidirectional promoter region *phoRP–phoU* showed a maximum expression level in phosphate-limited (40 μM) R5 medium at 24–27 h that decreases rapidly afterwards (Fig. 2), whereas expression of *pstS* was maximal at about 36 h and remained at a moderate expression level at 48 h. These results suggest that expression of both *phoR* and the modulator *phoU* genes are co-ordinately regulated, whereas expression of the phosphate transport *pstS* appears to follow a different pattern. The repression ratio (repressed versus derepressed reporter levels) was found to be ninefold for *phoRP*, 17-fold for *phoU* and 13-fold for *pstS*.

PhoP binds to three phosphate-regulated promoters

To study the PhoP interaction with the promoters, GST–PhoP fusion protein was expressed in *E. coli* and purified to near homogeneity (Fig. 3). To test whether the deletion of the PhoP N-terminal region (α5 helix) results in a higher affinity towards PHO boxes, as described in *E. coli* (Allen *et al*., 2001), a truncated version of PhoP was created and fused to GST. This fusion protein, GST–$PhoP^{DBD}$, contains the PhoP 115 last amino acids, starting at the Ile-109 residue. The alignment of *E. coli* PhoB and *S. coelicolor* PhoP proteins and the secondary structure described for PhoB (Solà *et al*., 1999; Okamura *et al*., 2000) are shown in Fig. 3. The Ile-109 amino acid is located into the α5 helix; therefore, the truncated PhoP protein at this position should have a similar behaviour to the truncated *E. coli* PhoB protein. The GST protein was expressed, purified and used in control experiments (Fig. 3B).

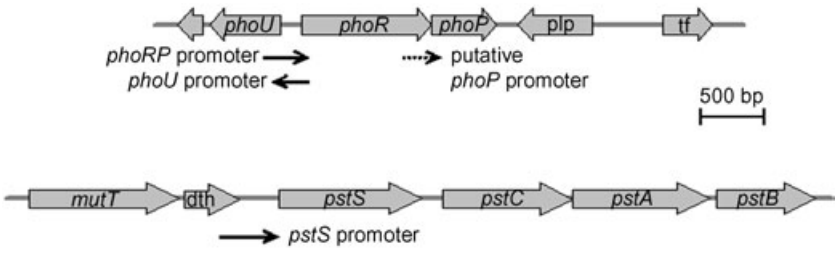


Fig. 1. Maps of the *S. coelicolor* chromosomal region containing the *phoRP–phoU* cluster and the *pstS* gene. The DNA fragments used as promoters of *pstS*, *phoRP* and *phoU* and the putative 'internal' *phoP* promoter are shown by thin arrows. *phoU*, phosphate signal transduction modulator; *phoR–phoP*, two-component system; plp, putative lipoprotein; tf, transcriptional factor; *mutT*, mutator gene; dth, diadenosine tetraphosphate hydrolase; *pst*, phosphate specific transport gene cluster.

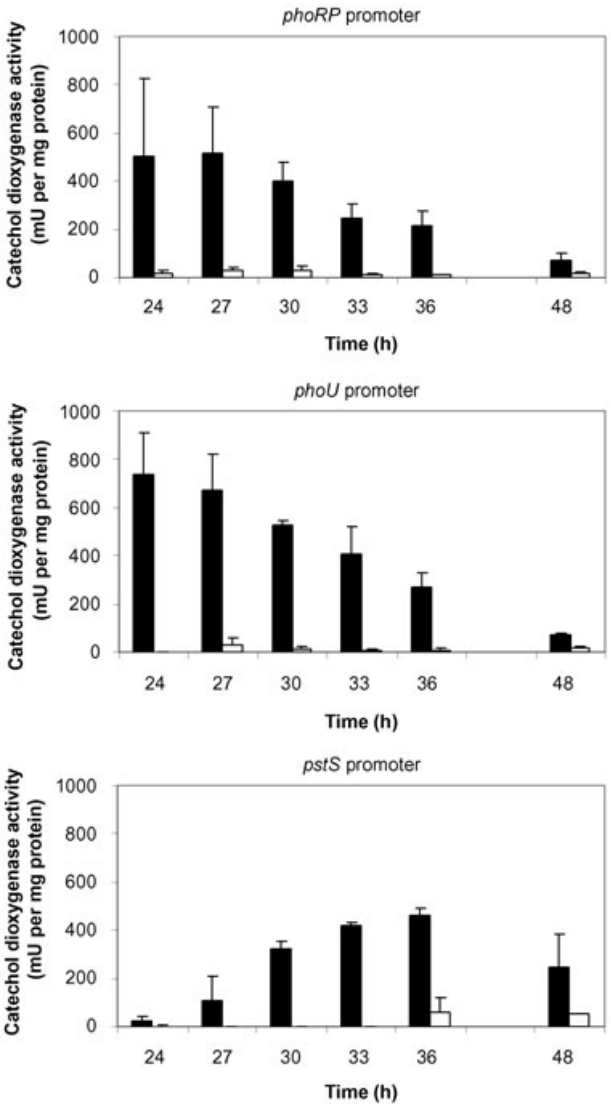


Fig. 2. Phosphate regulation of the promoter activity of *phoRP*, *phoU* and *pstS* using the *xylE* gene (encoding catechol dioxygenase) as reporter. The enzyme activity was measured at the indicated culture times. Note that there is a drastic reduction in promoter activity in phosphate supplemented (1.85 mM) (open bars) with respect to the control (40 μM; dark bars).

The promoter DNA–PhoP interaction was studied by electrophoretical mobility shift assays. When the *phoRP–phoU* bidirectional promoter region was used, at least two shifted bands were observed (Fig. 4A, arrows). No significant differences between GST–PhoP and GST–PhoP^{DBD} were found; the distinct mobility of these complexes resulted from the different protein size. Similarly, a shifted band was observed with the *pstS* promoter; a more intense shifting was observed at high GST–PhoP^{DBD} protein concentration (Fig. 4C). In both cases, control reactions made with pure GST protein were negative, excluding a possible binding of this protein to the promoters.

The existence of a putative *phoP* promoter (internal to the *phoR–phoP* cluster) was analysed using a *Nru*I restriction fragment, including the last 236 nucleotides of *phoR* and the first 82 nucleotides of *phoP* (Fig. 1). No band shift was observed, either with GST–PhoP or with GST–PhoP^{DBD} proteins (Fig. 4B), suggesting that there is not a phosphate-regulated separate promoter for the *phoP* gene.

Transcription start points of the pstS *and* phoU *promoters*

The origin of transcription of the three phosphate-regulated promoters was determined by primer extension. Results showed four transcription start points (*tsp*) in the *pstS* promoter, that were named *pstS*-P_1 to *pstS*-P_4, P_1 being the more distant and P_4 the closest to the ATG translation initiation triplet (Fig. 5).

When the *pstS* promoter region was compared (using the Patser search engine, see *Experimental procedures*) with the matrices described by Bourn and Babb (1995) that correspond to the *Streptomyces* –10 and –35 boxes (classes A and C respectively), we found that the –10 box with highest score to the consensus *Streptomyces* was TAATTT, located exactly 6 bp upstream of the *pstS*-P_1 transcription initiation site. A search using combined class C–class A matrices revealed a –35 box TCGGCG separated by 17 nucleotides distance, with a score of 3.87 for the 35 nt promoter sequence. Analysis of the upstream 50 nt of the putative *tsp pstS*-P_2 to *pstS*-P_4 produced low scores (0.76 for the upstream *pstS*-P_2 region, and 0.31 for the upstream *pstS*-P_3 region).

The bidirectional promoter region *phoRP–phoU* showed transcription initiation sites in both orientations, as shown by primer extension analysis. The *phoU* promoter showed a peak at a *tsp* located at 28–30 nucleotides upstream of the ATG translation initiation codon and other *tsp* at 71 nucleotides (named *phoU*-P_1; Fig. 6). Analysis of the region upstream of the *phoU*-P_1 revealed the presence of a –10 box GAACGT and a –35 box CGGAAA, with a score of 1.78 for the full promoter sequence. The signal at nucleotide 28–30 appears to be caused by premature termination of the primer extension at a hairpin formed in the mRNA in this region (see later, Fig. 8), a hypothesis that is supported by the variability of the nucleotide identified as +1 at the nucleotide 28–30 region (Fig. 6). Indeed, analysis of the region upstream of the putative *phoU*-P_2 did not produce any positive score.

phoR *is transcribed as a leaderless mRNA*

The *phoR* promoter showed a clear single *tsp* (Fig. 6) that was located at a G corresponding to the first putative ATG translation initiation codon. As there are two close possible translation initiation triplets (ATG and GTG separated

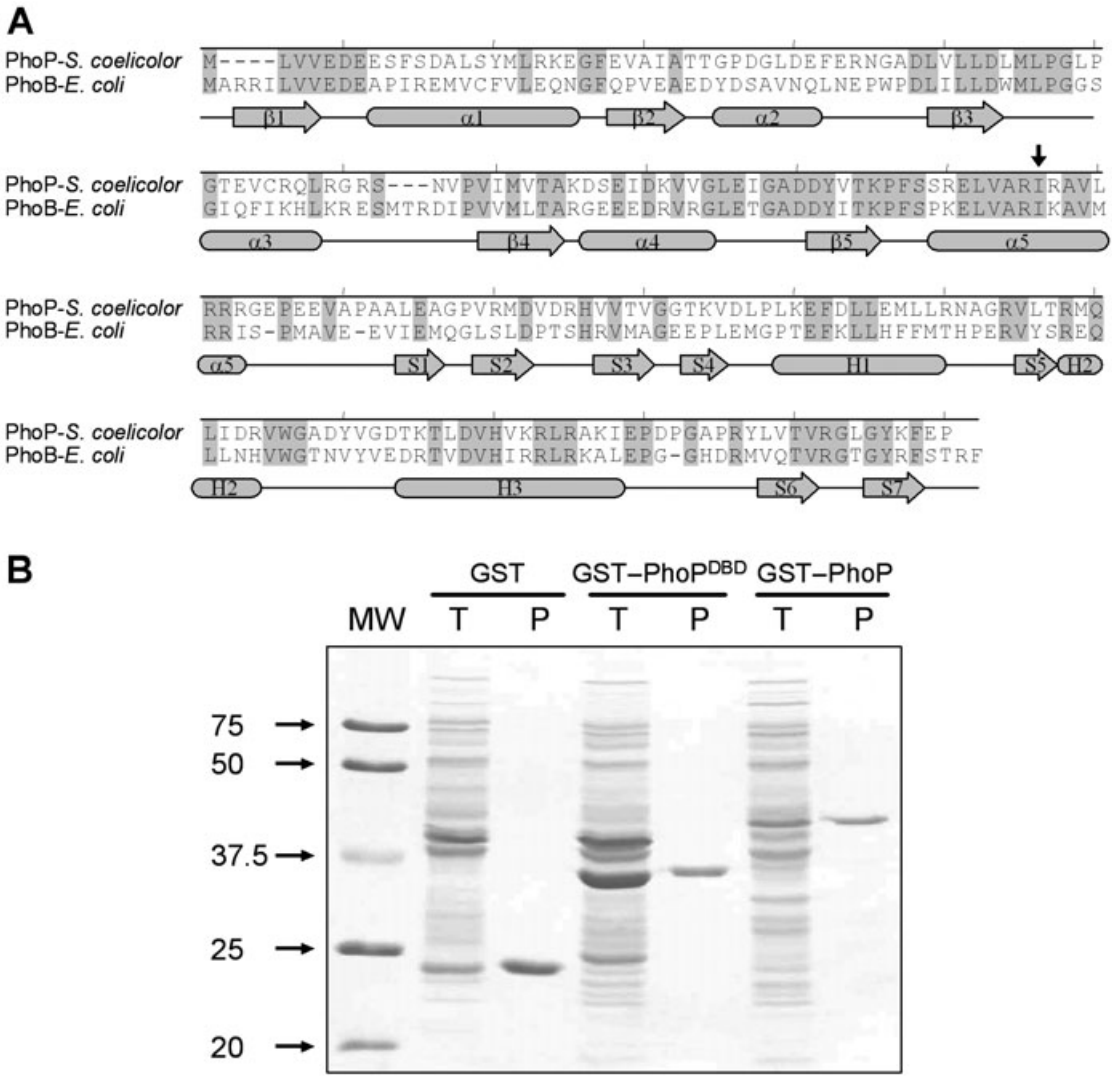


Fig. 3. A. Alignment of the conserved motifs in the *S. coelicolor* PhoP and *E. coli* PhoB proteins. The α-helices and β-sheets of the *E. coli* protein are shown. The name used for design the different structures is as described previously (Solà *et al*., 1999; Okamura *et al*., 2000). The thick vertical arrow indicates the position at which the PhoP protein is truncated to obtain the PhoP-DBD domain (extending from Ile-109 to the end of the protein).
B. Purification of GST (as a control), GST–PhoP^{DBD} and GST–PhoP proteins by affinity chromatography on Glutathione Sepharose. Lane T, total *E. coli* cell extract; lane P, purified proteins after affinity chromatography. Left lane, molecular size markers (in kDa; arrows).

by three nucleotides), these results suggest that the GTG (a frequent translation initiation codon in *Streptomyces*) is the first translated triplet. The Patser analysis of the upstream sequence using the matrices of Bourn and Babb revealed a clear promoter with a score of 5.68. The −10 box of this promoter is TAACCT, located 8 nt upstream of the observed *tsp*, and the −35 box (TGGGGC) is separated by 18 nt. These results suggest that the starts of transcription and translation practically coincide in *phoR*, as reported for the leaderless mRNA of genes belonging to another two-component systems such as *afsQ1–afsQ2* (Ishizuka *et al*., 1992; Hutchings *et al*., 2004) and *vanR–vanS* (Hong *et al*., 2004).

DNase I protection studies reveal binding sites in pstS *and* phoU *promoters*

To determine the PhoP binding sequences, the bidirectional *phoRP–phoU* promoter region and the separate *pstS* promoter were studied by DNase I protection analysis.

Increasing concentrations of GST–PhoP^{DBD} protein from 0.03 μM to 1.1 μM were tested using 5′-end fluorescein-labelled DNA fragments, as described previously (Rodríguez-García *et al*., 1997). Results of the analysis of the PST-CO fragment showed a protected region extending for 29 bp of the coding strand of the *pstS* promoter. Full protection of this nucleotide stretch was achieved at

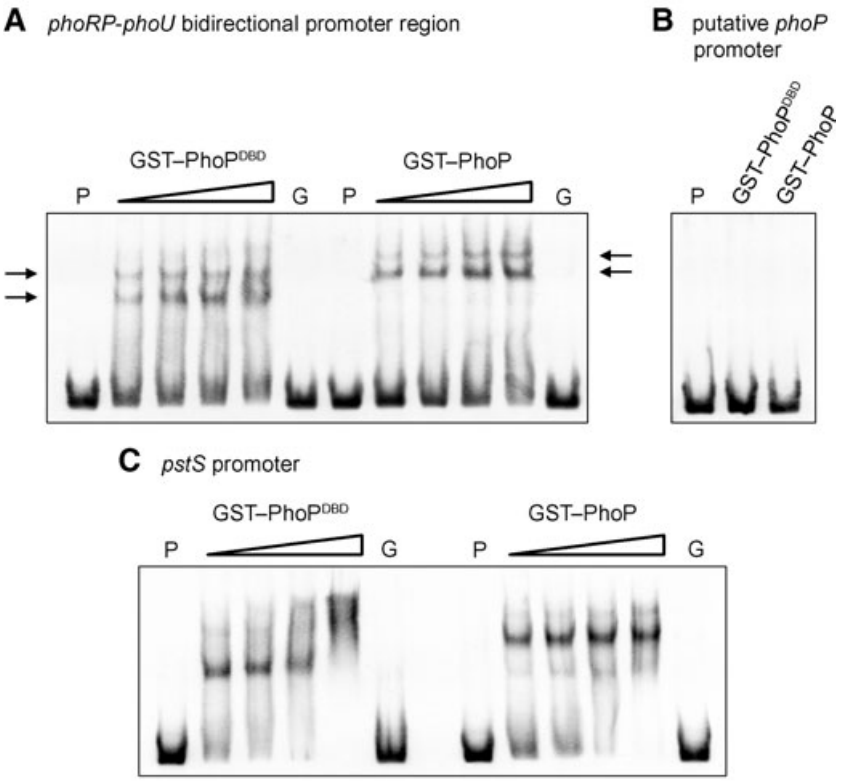


Fig. 4. Electrophoretic mobility shift assays of the *phoRP–phoU* bidirectional promoter region (A), the putative *phoP* (internal) promoter (B) and the *pstS* promoter (C) using increasing concentrations (3.12, 6.25, 12.5 and 25 pmols) of the GST–PhoP^{DBD} (truncated DBD domain) and GST–PhoP (complete protein). Lane G, control with pure GST protein (150 pmols). Lane P, probe without protein. The two shift bands of the *phoRP–phoU* bidirectional promoter region (A) are indicated by arrows.

very low (0.07 μM) GST–PhoP^{DBD} concentration and was not enlarged by increasing protein concentrations (Fig. 7A). This protected region is located at nucleotide positions –66 to –38 with respect to the *pstS*-P_1 transcription start site. The protection of the reverse strand of the *pstS* promoter showed the same requirement for GST–PhoP^{DBD} protein (0.07 μM; PST-RE fragment). In this strand, the protection is three nucleotides shorter than that of the coding strand from the distal end (positions –63 to –38; Fig. 7B).

In the protected *pstS* region there are two 11-nucleotide sequences that form a direct repeat. Each direct repeat unit (DRu) is formed by a five-nucleotide sequence that is identical between both units (GTTCA), followed by a six-nucleotide less-conserved tail (Fig. 8A). These direct repeat units, named DRu-S1 and DRu-S2, reminds the *E. coli* PHO box that is composed by two direct repeat units of 11 nucleotides that include eight well-conserved nucleotides and a tail of three more variable ones (Blanco *et al.*, 2002).

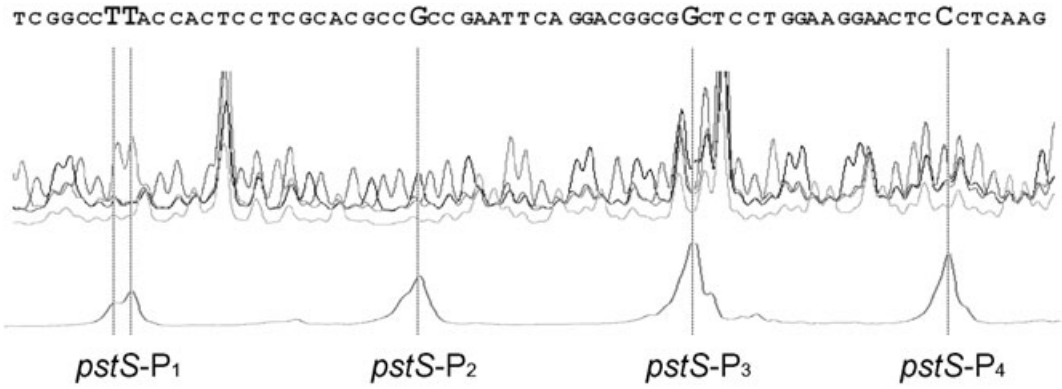


Fig. 5. Primer extension analysis of the promoter region upstream of the *pstS* gene. Note the presence of four peaks in the primer extension reaction product (*pstS*-P_1 to *pstS*-P_4) (lower fluorogram). The full nucleotide sequence is shown in the upper fluorogram (see Fig. 8 for details of the nucleotide sequences). The nucleotides corresponding to ends of the primer extension are highlighted with capital letters. RNA was extracted from strains containing the *xylE*-coupled promoter in a high-copy-number plasmid for a better resolution. Cells were grown in R5 liquid medium with low phosphate concentration (40 μM) for 30 h, time at which a high cathecol oxygenase activity was detected.

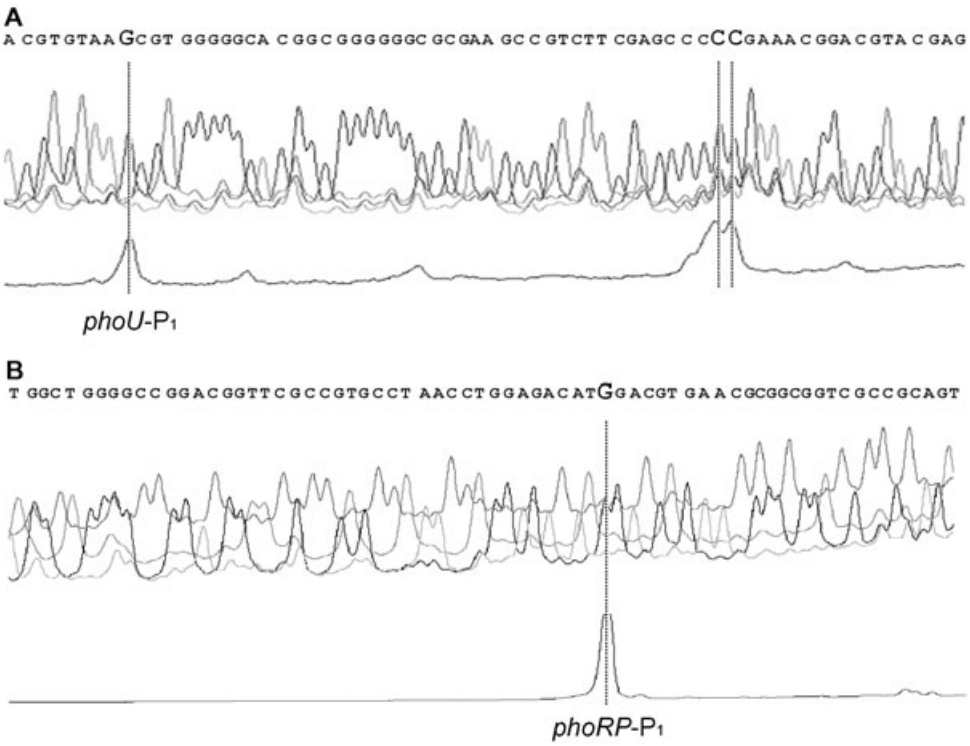


Fig. 6. Primer extension analysis of the *phoU* promoter (A) and the *phoRP* promoter (B) regions. Note that there are two transcription initiation points (see text) in the *phoU* promoter and only one in the *phoRP* promoter (see Fig. 8 for details of the nucleotide sequence). RNA extraction and primer extension conditions were as indicated in the legend to Fig. 5.

Footprinting assays of the *phoU* promoter revealed a 51-nucleotide protection in the coding strand (positions −89 to −39 with respect to the *phoU*-P_1 transcription start site; Fig. 9A). As occurs in the *pstS* promoter, in the bottom strand the protected sequence was slightly shorter (46 nucleotides; positions −83 to −38). A concentration of 0.53 μM of GST–$PhoP^{DBD}$ was necessary to achieve full protection in both strands, i.e. near 10 times higher than that required for the *pstS* binding site protection (Fig. 9B).

This region contains four 11-nucleotide consecutive DRu (Fig. 8B). Two of them agree well with the consensus (DRu-U2 and DRu-U4), and the other two are more variable (DRu-U1 and DRu-U3). The length of the protected region (51 nt, top strand) clearly indicates that the DRu-U1 and DRu-U3 are also bound by PhoP, even though they showed only partial conservation. The protected region in the *phoU* promoter therefore corresponds to two full PHO boxes (2 × 22 bp) and each PHO box consists of two DRu of 11 bp. The presence of two PHO boxes in tandem has also been reported in *E. coli* (Kimura *et al*., 1989; Kasahara *et al*., 1991; Kim *et al*., 2000).

Discussion

No molecular information was available so far on the phosphate control sequences in the phosphate-regulated genes of *Streptomyces* species (Martín, 2004). As described in this article, inorganic phosphate strongly represses the expression of the reporter *xylE* gene when coupled to the *pstS* promoter or to the intergenic *phoRP–phoU* region in both orientations. The repression was particularly intense for the *phoU* and *pstS* promoters in good correlation with the presence of the PHO boxes in these promoters.

Electrophoretic mobility shift assays proved that both the complete *S. coelicolor* PhoP protein and the truncated DBD domain bind with high affinity to the promoter regions of the three phosphate-regulated genes. Similar results were observed in *E. coli* when the truncated $PhoB^{DBD}$ domain was used (Ellison and McCleary, 2000). More than one shift band was obtained with the *phoRP–phoU* promoter region at increasing protein concentrations, a phenomenon observed also in the binding of other *S. coelicolor* regulatory proteins such as DmdR1 and DmdR2 (Flores and Martín, 2004). The formation of two complexes of different molecular weight indicates that more than one binding site is present in the *phoRP–phoU* promoter region, a result that was confirmed by the footprinting studies.

Footprinting analysis revealed a protected sequence of 29 nucleotides in the *pstS* promoter encompassing two direct repeat units, each of 11 base pairs. The protected

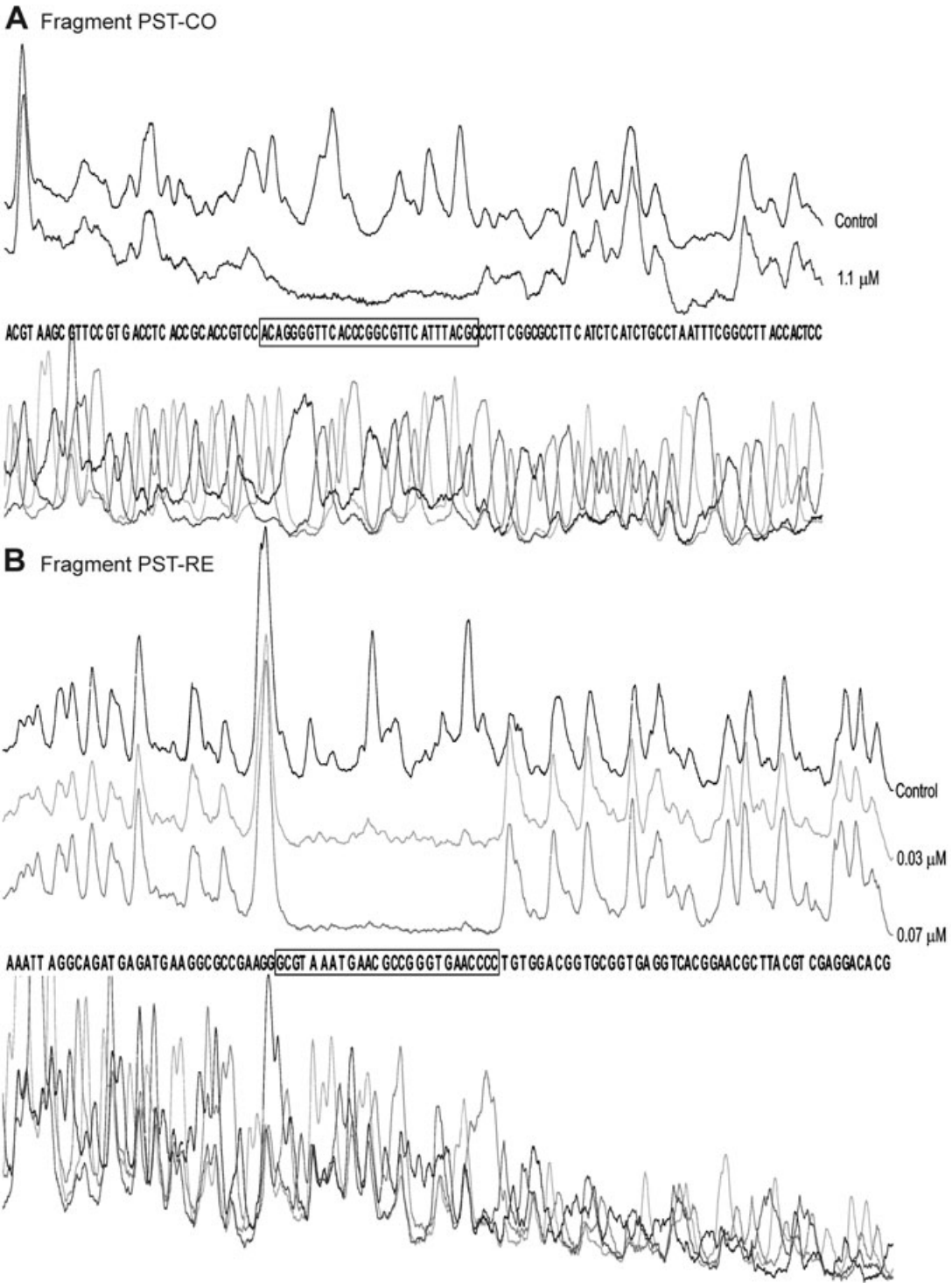
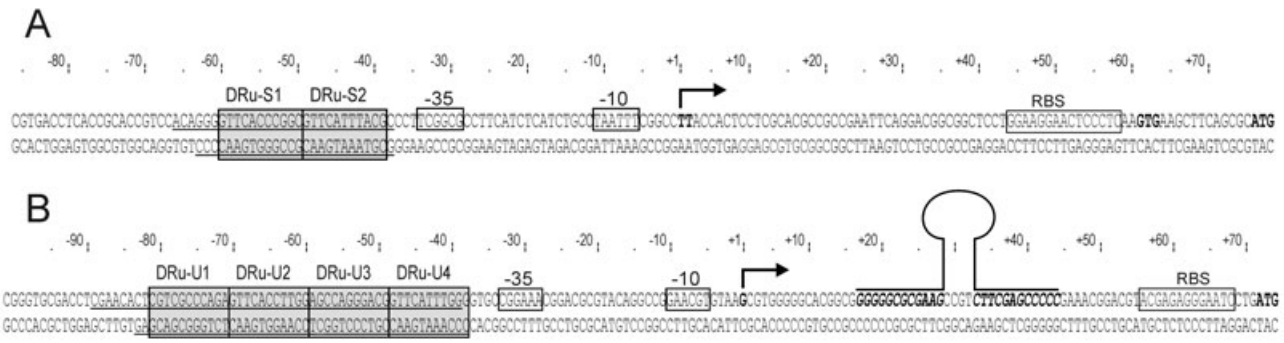


Fig. 7. Footprinting of the *pstS* promoter region using PhoP^{DBD} protein.
A. PST-CO DNA fragment (coding strand).
B. PST-RE DNA fragment (reverse strand).
The upper fluorogram corresponds to the control DNA fragment and to the protected reactions and the lower one to the full nucleotide sequence. The protected nucleotide sequence is boxed. Protein concentration was 1.1 μM for the sense strand (A), 0.03 and 0.07 μM for the reverse strand (B).



C

	1	2	3	4	5	6	7	8	9	10	11
DRu-S1	G	T	T	C	A	C	C	C	G	G	C
DRu-S2	G	T	T	C	A	T	T	T	A	C	G
DRu-U1	C	G	T	C	G	C	C	C	A	G	A
DRu-U2	G	T	T	C	A	C	C	T	T	G	G
DRu-U3	A	G	C	C	A	G	G	G	A	C	G
DRu-U4	G	T	T	C	A	T	T	T	G	G	G
A	17%	0%	0%	0%	83%	0%	0%	0%	50%	0%	17%
C	17%	0%	17%	100%	0%	50%	50%	33%	0%	33%	17%
G	67%	33%	0%	0%	17%	17%	17%	17%	33%	67%	67%
T	0%	67%	83%	0%	0%	33%	33%	50%	17%	0%	0%
	G	**G/T**	**T**	**C**	**A**	**Y**	**Y**	**Y**	**R**	**C/G**	**G**

Fig. 8. A and B. Phosphate boxes in the *pstS* promoter (A) and in the *phoU* promoter (B). The direct repeat units (DRu in each PHO sequence) are indicated with shaded boxes. The −10 and −35 hexanucleotides are boxed. Nucleotides showing homology with the 16S RNA that could form a ribosomal binding site are framed with a box labelled RBS. The first triplet is shown in boldface letters. The transcription start points are indicated by bent arrows. A putative stem and loop structure in the *phoU* promoter is indicated.
C. Matrix summarizing the frequencies of each nucleotide in the DRu existing in the protected regions (PHO boxes). The consensus PHO box is shown in boldface letters in the lower line.

sequence in the *phoRP–phoU* promoter region was of 51 nucleotides in the *phoU* sense strand, and it covered 46 coincident nucleotides in the complementary strand (*phoR* sense) including four repeat units (DRu-U1 to DRu-U4). Two of these sequences in the *phoR–phoU* intergenic region were very similar to the DRu of the *pstS* binding site. Both these sequences, DRu-U2 and DRu-U4 (Fig. 9), lie in the sense strand of *phoU* and are included in the PhoP-protected region. In *E. coli* the union of the DNA-binding PhoB protein is asymmetric and is oriented towards the activated promoter (Blanco *et al*., 2002). It is very likely that the same occurs in *S. coelicolor*, and consequently, the *phoU* expression will be induced when PhoP is phosphorylated.

We conclude therefore that in *S. coelicolor* each PHO box consists of two tandem direct repeat units of 11 bp. In *E. coli* PHO boxes have been described as 18 bp DNA sequences (Makino *et al*., 1994). However, Blanco *et al*. (2002) reported after analysis of the crystal structure of the $PhoB^{DBD}$–DNA complex that a single PhoB monomer recognizes a direct repeat unit of 11 bases, in agreement with the 11 bp DRu observed in this work. The footprinting studies of Kim *et al*. (2000) show that the 48 nt protected region of the *E. coli psiE* promoter extends three nucleotides upstream of the first PHO box and finishes with the last nucleotide of the second PHO box in agreement with our findings in *S. coelicolor*.

The alignment of the six *S. coelicolor* direct repeat units shown in this article to be protected by PhoP and the frequencies of nucleotides in each position of the 11-mer sequence is shown in Fig. 8C. Results showed that the first five positions are highly conserved: position 4 is always a C, and positions 3 and 5 are always a T and an A, respectively, except in one repeat. The consensus sequence deduced from this alignment is $^{1}G^{G/T}TCAYYYR^{G/C}G^{11}$, where Y is a pyrimidine and R a purine. Similar PHO boxes have been found in the homologous genes of other *Streptomyces* species (A. Rodríguez-García *et al*., unpublished). The repeats DRu-U2 and DRu-U4 of the *phoU* promoter and DRu-S1 and DRu-S2 of the *pstS* promoter closely matched the PHO box consensus, whereas DRu-U1 and DRu-U3 were less conserved.

In the *phoRP–phoU* bidirectional promoter we have shown that the PhoP protein binds four consecutive direct

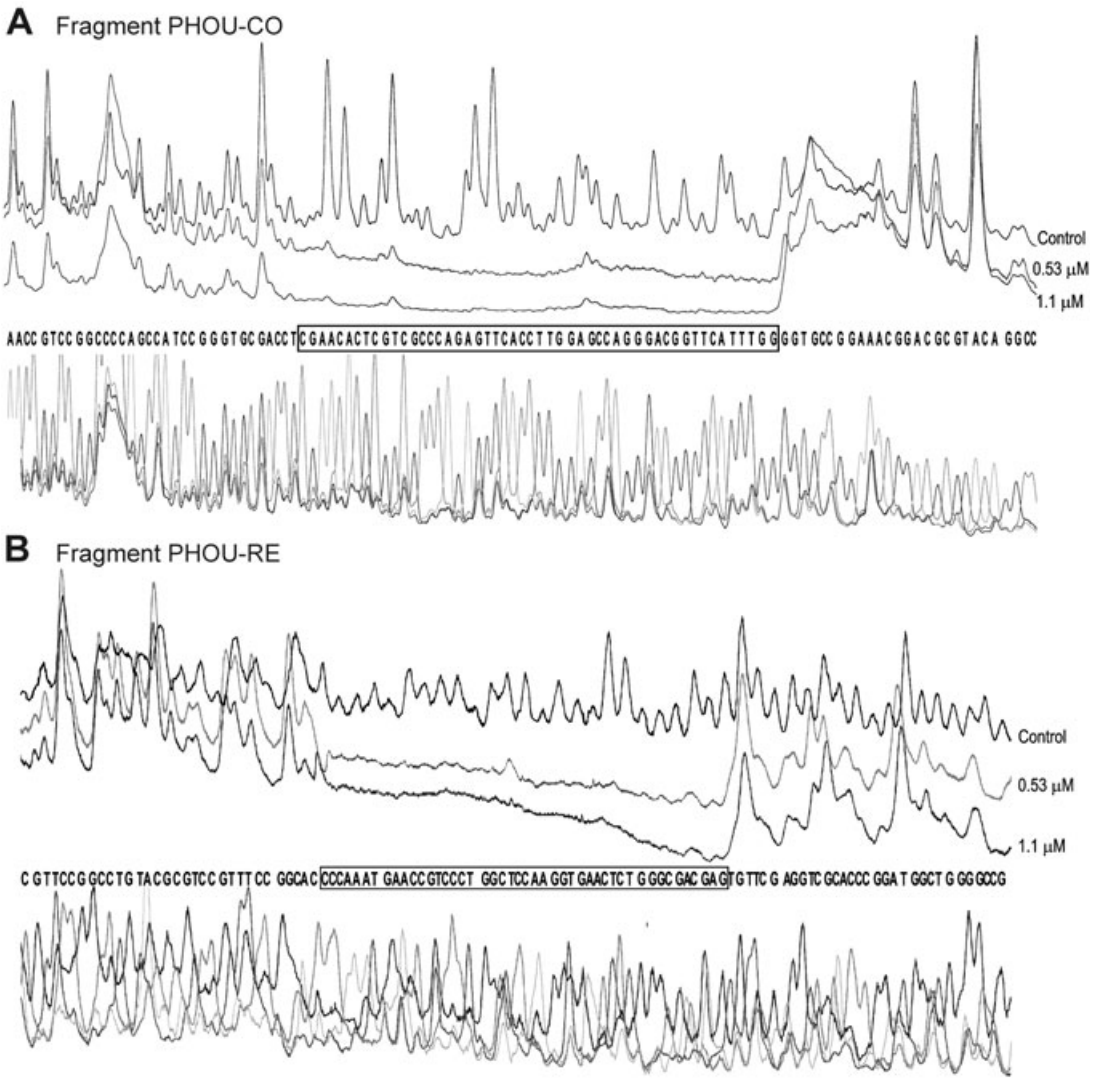


Fig. 9. Footprinting of the *phoU* promoter region using PhoP^{DBD} protein.
A. Footprinting of the PHOU-CO DNA fragment (coding strand of the *phoU* gene).
B. Footprinting of the PHOU-RE DNA fragment (reverse strand of the *phoU* gene).
Only the results of two concentrations (0.53 and 1.1 μM) of PhoP^{DBD} protein are shown. Note the protection of a 51 nt stretch (boxed) in the coding strand.

repeat units, that form two PHO boxes. Two or more tandemly arranged PHO boxes (each consisting of two direct repeat units) have also been reported in *E. coli*, e.g. in the promoters of the *ugp* operon (Kasahara *et al*., 1991), the *pst* operon (Kimura *et al*., 1989) and the *psiE* promoter (Kim *et al*., 2000).

The electrophoretic mobility shift assays (EMSA) of *phoRP–phoU* promoter region showed two shifted bands. The lower band is likely the result of binding one PhoP-dimer to one DRu pair, and the upper band probably results from the binding of a tetramer of PhoP to the four DRu. The fact that the GST–PhoP^{DBD} requirement to obtain full protection in the *phoU* promoter is near 10 times higher than that of the *pstS* promoter correlates with the lower conservation of the PHO boxes found in the *phoU* promoter. In the *pstS* case, two well-conserved DRu allow a strong interaction between a dimer of PhoP and the DNA. However, in the *phoU* promoter two DRu showing lower conservation are intercalated between the two conserved DRu. This implies that more protein is needed to stabilize the interaction of a PhoP dimer to two consecutive DRu.

Experimental procedures

Bacterial strains and plasmids

The bacterial strains and plasmids used in this work are listed

Table 1. Bacterial strains and plasmids.

Strains/plasmids	Characteristics	Reference
Strains		
Streptomyces lividans JI 1326	Wild type	John Innes[a]
S. coelicolor M145	Wild type	Kieser *et al*. (2000)
Escherichia coli DH5α	F′ ϕ80 *dLacZ* ΔM15 Δ(*lacZYA-argF*) *U16 recA1 endA1 hsdR17* ($r^- m^+$) *supE44 medλ*$^-$ *thi-1 gyrA relA1*	Hanahan (1983)
Plasmids		
pBluescript KS+	Cloning vector, ColE1 origin, Amp^r	Stratagene
pBS*phoRP*	*phoU* (partial), *phoR* and *phoP* genes cloned into pBluescript KS+	Sola-Landa *et al*. (2003)
pBS-P*phoRP*	*Hin*cII–*Sca*I fragment carrying *phoRP* promoter cloned into pBluescript KS+ (*Eco*RV)	This work
pBS-P*phoU*	*Pvu*I fragment carrying *pho*U promoter cloned into pBluescript KS+ (*Eco*RV)	This work
pGEM-T-easy	Vector system for the cloning of PCR products	Promega
pGEM-P*pstS*	PCR product carrying *pst*S promoter cloned into pGEM-T-easy	This work
pIJ4083	Promoter-probe plasmid using *xylE* as reporter	Clayton and Bibb (1990)
pIJ-P*phoRP*	*phoRP* promoter cloned into pIJ4083	This work
pIJ-P*phoU*	*phoU* promoter cloned into pIJ4083	This work
pIJ-P*pstS*	*pstS* promoter cloned into pIJ4083	This work
pGEX-2T	Vector system for protein expression	Pharmacia Biotech
pGEX-PhoP	*S. coelicolor phoP* gene cloned into pGEX-2T	This work
pGEX-DBD	*S. coelicolor phoP*-DBD gene cloned into pGEX-2T	This work

a. Collection of microorganisms of the John Innes Institute, Colney Lane, Norwich NR4 UH, UK.

in Table 1. The *phoRP* promoter was subcloned from pBS*phoRP* (Sola-Landa *et al*., 2003) as a *Hin*cII–*Sca*I fragment into the *Eco*RV site of pBluescript KS+. This plasmid, named pBS-P*phoRP*, carries the 370 bp upstream of *phoR* and the first 47 bp of the *phoR* coding region. Similarly, the *phoU* promoter was cloned as a *Pvu*I fragment from pBS*phoRP*, the ends blunted with the Klenow fragment of DNA polymerase and subcloned into the *Eco*RV site of pBluescript KS+. This plasmid, named pBS-P*phoU*, carries the upstream region (250 bp) of *phoU* and the first 33 bp of *phoU* coding region.

The *pstS* promoter was cloned by polymerase chain reaction (PCR) with oligonucleotides O1-ACGAGGATCCCCG CATCCTGTGGCTG and O2-CCCGTCTAGACATATGCT GAAGCTTCACTTG as primers and cloned into pGEM-T-easy (Promega), obtaining pGEM-P*pstS*. The constructions were sequenced using pUC/M13 Forward and Reverse primers in a Perkin Elmer ABI Prism 310 Genetic Analyzer. Plasmids pIJ-P*phoRP* and pIJ-P*phoU* contain the *Hin*dIII–*Bam*HI fragments from pBS-P*phoRP* and pBS-P*phoU*, respectively, and plasmid pIJ-P*pstS* the *Bam*HI–*Xba*I fragment from pGEM-P*pstS*, cloned into the *Streptomyces* vector pIJ4083 (Clayton and Bibb, 1990).

To express the different proteins in *E. coli* the *phoP* gene was amplified by PCR using the O3-TTCCGTGgatccGT GCTCGTCGT, and O5-GGGGAAGCTTACGGCTCAAACT TGT primers (where the *Bam*HI restriction site is shown in lower case letter) and cloned into pGEM-T-easy (Promega). The insert was extracted by digestion with *Bam*HI and *Eco*RI (the latter site from the vector) and was cloned into pGEX-2T expression plasmid (Pharmacia Biotech). The new pGEX-PhoP plasmid was introduced in *E. coli* DH5α. Similarly, plasmid pGEX-DBD was constructed using O4-CTGGTGgatccATCCGAGCCGTACT and O5 primers. The correct integration of inserts was established by sequencing.

Quantification of the promoter activity using the reporter xylE *gene*

For promoter activity studies approximately 3×10^6 *S. coelicolor* spores were pre-germinated in 2× YT liquid medium for 8 h at 30°C. Germinated spores were harvested by centrifugation, resuspended in water and used to inoculate 100 ml of R5 liquid medium. Thiostrepton was added at final concentration of 5 μg ml^{-1} as selective antibiotic. Catechol-2,3-dioxygenase activity of the *xylE* reporter gene was measured as described by Kieser *et al*. (2000).

Protein expression and purification

To obtain the fusion proteins, *E. coli* cells were grown in LB medium at 25°C in an orbital shaker (250 r.p.m.) to an OD_{600} of 0.6. Expression was induced with IPTG (0.1 mM final concentration) for 5 h. Cells were harvested by centrifugation, washed twice with NaCl 0.9% and lised by sonication. Soluble fractions were separated by centrifugation and proteins were purified in an ACTA-FPLC using a Glutathione Sepharose 4B column (Pharmacia Biotech). Proteins were eluted with 10 mM reduced glutathione (in 50 mM Tris·HCl, pH 8.0) following the manufacturer's recommendations and conserved in 40% glycerol at –80°C before use. Protein concentration was determined with the Bradford reagent (Bio-Rad).

DNA–protein binding assays

DNA binding tests were performed by the EMSA. The DNA fragments were labelled at both ends with digoxigenin with DIG Oligonucleotide 3′-End Labeling Kit, 2nd Generation (Roche Applied Science). The binding reaction contained 10 mM Tris·HCl pH 8.0, 0.4 mM $MgCl_2$, 10 mM KCl, 0.2 mM

DTT, 1.6 mM glutation, 0.01% Nonidet P40, 50 μg ml^{-1} poly[d(I-C)], 13% glycerol. The reaction was performed as described for *Mycobacterium bovis* RegX3 protein (Himpens *et al*., 2000), which has a 72.7% identity with *S. coelicolor* PhoP. The samples were loaded onto a 5% polyacrylamide (29:1) native gel in 0.5× TBE buffer. After electrophoresis (5 h, 80 V), DNA was electroblotted onto a nylon membrane in 0.5× TBE buffer (1 h, 200 mA). The DNA was fixed by UV cross-linking, detected with anti-digoxigenin antibodies and developed by chemiluminiscence with the CDP-Star™ reagent (Roche Applied Science).

Footprinting assays

DNase I footprinting assays were performed by the fluorescent labelling procedure (Rodríguez-García *et al*., 1997), using the GST–PhoP^{DBD} protein form that has higher affinity than the complete PhoP protein. DNA fragments of the intergenic *phoRP–phoU* bidirectional promoter and the *pstS* promoter were obtained by PCR using M13-20 and reverse primers, one of them fluorescein labelled. pBS-P*phoU* and pGEM-P*pstS*, respectively, were used as template DNAs. In each case, the same labelled oligonucleotide served to prime the sequencing reaction used as the molecular size marker. The PCR products were purified after agarose-gel electrophoresis and DNA concentrations were determined with a GeneQuant™ spectrophotometer (Amersham Biosciences). The labelled fragments were designated as follows, according to the labelled strand: (i) PHOU-CO (401 bp), coding strand of the *phoU* promoter (this is therefore the reverse of *phoR*), (ii) PHOU-RE (514 bp), reverse strand of the *phoU* gene, (iii) PST-CO (546 bp), coding strand of *pstS* promoter and (iv) PST-RE (550 bp), reverse strand of *pstS* promoter.

The reaction components were the same as described above for the EMSA reaction. Labelled DNA fragment (0.28 pmol) and GST–PhoP^{DBD} protein were added to a final volume of 28 μl, and incubated at 30°C for 30 min. Lyophilized bovine pancreas DNase I (Roche grade I) was reconstituted in 20 mM Tris·HCl pH 7.0, 50 mM NaCl, 100 μg ml^{-1} BSA, 1 mM DTT, 50% glycerol to a final concentration of 20 units ml^{-1}. Further dilutions were made in the same solution supplemented with 10% glycerol. Nuclease digestions were carried out with 2 μl of the 1:8000 dilution (5×10^{-3} units) at 30°C for 1 min and stopped with 120 μl of 40 mM EDTA in 9 mM Tris·HCl pH 8.0. After phenol-chloroform purification and ethanol precipitation, samples were loaded in an ALF DNA sequencer (Amersham Biosciences). Results were analysed with the FRAGMENT MANAGER program (Amersham Biosciences).

RNA procedures and primer extension

Streptomyces RNA was isolated by the hot phenol procedure adapted to *Streptomyces* (Patek *et al*., 2003). The transcriptional start sites were determined by primer extension using the 5′-fluorescein-labelled oligonucleotide O6-GCGATCGCT GCCACTGC as primer (complementary to the 5′-coding region of *xylE* from pIJ4083 vector). The reaction was loaded in an ALF DNA sequencer (as indicated above) and analysed with the FRAGMENT MANAGER program.

Bioinformatic analysis

The candidate sequences to contain promoters were analysed using the Patser algorithm (Hertz and Stormo, 1999), implemented in the web resource Regulatory Sequence Analysis Tools (van Helden, 2003). The pseudocount value was set to 10, and the alphabet parameter was adjusted to the GC content of *Streptomyces* genome: AT, 0.15; CG, 0.35. The matrices used to search for regions −35 and −10 were those derived from the alignments of class C and class A promoters of Bourn and Babb (1995). To search for a combination of 'class C–*n* nt of separation–class A', we included *n* columns of null values in the combined matrix.

Acknowledgements

This work was supported by Grants BIO2003-01489 and GEN2003-20245-C09-01-NAC of the CICYT, Ministry of Science and Technology, Madrid, Spain. E.F.-D. received a fellowship of the Diputación de León, Spain. We thank J. Merino, B. Martín, A. Casenave and M. Álvarez for the excellent technical assistance.

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