Phosphate control of \(\text{phoA} \), \(\text{phoC} \) and \(\text{phoD} \) gene expression in \(\text{Streptomyces coelicolor} \) reveals significant differences in binding of PhoP to their promoter regions

Alexander K. Apel, Alberto Sola-Landa, Antonio Rodríguez-García and Juan F. Martín

Correspondence
Juan Francisco Martín
jf.martin@unileon.es

1 Instituto de Biotecnología de León, INBIOTEC, Parque Científico de León, Av. Real 1, 24006 León, Spain
2 Área de Microbiología, Fac. CC. Biológicas y Ambientales, Universidad de León, Campus de Vegazana, s/n, 24071, León, Spain

Three putative alkaline phosphatase genes, \(\text{phoA} \), \(\text{phoC} \) and \(\text{phoD} \), were identified in the genome of \(\text{Streptomyces coelicolor} \) by homology with the amino acid sequence obtained from the PhoA protein of \(\text{Streptomyces griseus} \). PhoA and PhoC correspond to broad-spectrum alkaline phosphatases whereas PhoD is similar to a \(\text{Ca}^{2+}\)-dependent phospholipase D of \(\text{Streptomyces chromofuscus} \). The \(\text{phoA} \) and \(\text{phoD} \) genes were efficiently expressed in R5 medium under phosphate-limited conditions, as shown by studies using the \(\text{xylE} \) reporter gene, whereas \(\text{phoC} \) was poorly transcribed under the same conditions. Expression of \(\text{phoA} \) was clearly PhoP-dependent since it was not transcribed in the \(\text{S. coelicolor} \) \(\Delta\text{phoP} \) mutant and was strongly activated under low phosphate concentrations. Similarly, expression of \(\text{phoD} \) was PhoP-dependent and highly sensitive to phosphate availability. By contrast, expression of \(\text{phoC} \) was not PhoP-dependent. Electrophoretic mobility shift assays showed that PhoP binds to the \(\text{phoA} \) and \(\text{phoD} \) promoters, but not to that of \(\text{phoC} \). Footprinting studies with GST–PhoP revealed the presence of a PHO box (two direct 11 nt repeats) in the \(\text{phoA} \) promoter and two PHO boxes in the promoter of \(\text{phoD} \). The transcription start points of the three promoters were identified by primer extension. The transcription start point of \(\text{phoD} \) coincides with the G of its translation start codon, indicating that this gene is transcribed as a leaderless mRNA. The deduced –10 and –35 regions of \(\text{phoD} \) (but not those of \(\text{phoA} \)) overlapped with the PHO boxes in this promoter, suggesting that an excess of PhoP interferes with binding of the RNA polymerase to this promoter. In summary, the three promoters showed clear differences in the modulation of their expression by PhoP.

INTRODUCTION

\(\text{Streptomyces} \) species are soil dwelling micro-organisms that secrete many extracellular enzymes to degrade polymeric substrates and to utilize the nutrients obtained after their hydrolysis (García-González et al., 1991; Fernández-Ábalos et al., 1992; Schaerlaekens et al., 2004). Phosphate is an essential nutrient for growth of all micro-organisms. \(\text{Streptomyces} \) species and other soil micro-organisms may use inorganic phosphate, which occurs frequently in the form of partially insoluble phosphate salts, and organic phosphate, which is present in a variety of plant materials such as phytic acid (Martínez-Dominguez et al., 2002), sugar phosphates and nucleotides (Martin & Demain, 1977). To obtain phosphate from organic compounds, \(\text{Streptomyces} \) and other bacteria use an extracellular alkaline phosphatase encoded by the \(\text{phaA} \) gene (Moura et al., 2001). When the \(\text{Streptomyces griseus} \) \(\text{phaA} \) gene was cloned and compared with the genome of \(\text{Streptomyces coelicolor} \), two other putative phosphatase genes (hereafter named \(\text{phoC} \) and \(\text{phoD} \); \(\text{phoB} \) was avoided because this designation is used for the response regulator in \(\text{Escherichia coli} \), in addition to the \(\text{phaA} \) orthologue, were found (Moura et al., 2001; Bentley et al., 2002). Different phosphatases may work on different phosphorylated substrates.

Species of the genus \(\text{Streptomyces} \) are the major producers of secondary metabolites, including antibiotics and other pharmacologically active compounds, pigments, toxins, plant growth factors, etc. (von Döhren & Gräfe, 1997; Demain & Fang, 2000; Martin et al., 2000). The

Abbreviations: EMSA, electrophoretic mobility shift assay; DBD, DNA-binding domain; GST, glutathione S-transferase.
biosynthesis of most of these secondary metabolites is negatively controlled by phosphate and their production occurs only under phosphate-limited conditions (Martin & Demain, 1980; Martin et al., 1994).

Expression of phosphate-regulated genes is modulated by the two-component system PhoR–PhoP (Sola-Landa et al., 2003; Ghorbel et al., 2006). Binding of the response regulator PhoP to the promoters of phosphate-regulated genes was shown to modulate expression of primary metabolism genes, including phoA, by Western blot analysis (Sola-Landa et al., 2003), and genes involved in actinorhodin and undecylprodigiosin biosynthesis. Binding of the response regulator PhoP to the promoter regions of three genes of the pho regulon, pstS, phoU and phoRP, was shown both in S. coelicolor (Sola-Landa et al., 2005) and Streptomyces natalensis (Mendes et al., 2007), but direct binding of PhoP to the promoters of secondary metabolite biosynthesis genes has not been reported (Martin, 2004).

Synthesis of the PhoA alkaline phosphatase, as shown by Western blot analysis, is regulated positively by PhoP since in deletion mutants (either ΔphoP or ΔphoRP) this protein is not detected (Sola-Landa et al., 2003). However, it is not known whether the two-component system exerts its action by direct binding of PhoP to specific sequences in the phoA promoter region or whether regulation proceeds through other DNA-binding proteins by a cascade mechanism. Moreover, it is not known if the different phosphatase genes are regulated or not by phosphate availability or whether they respond to the same or to different type(s) of phosphate control.

It was, therefore, important to perform a comparative study of the mechanism of phosphate control of phoA, phoC and phoD expression using DNA electrophoretic mobility shift assays (EMSAs) as well as reporter gene expression.

In this article we report significant differences in the control of the three putative phosphatase-encoding genes. EMSA and footprinting analyses provided evidence for the presence of PhoP-binding sequences (PHO boxes) in the promoters of phoA and phoD but not in that of phoC, which is regulated by a different mechanism.

**METHODS**

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this work are listed in Table 1. Oligonucleotides were designed to amplify 500 bp fragments of the promoters of phoA, phoC and phoD containing sites for the restriction enzymes HindIII in the forward (F) and BglII or BamHI in the reverse (R) primers (underlined in the sequences below).

The phoA promoter was cloned from cosmid SCC75A by PCR with the primer pair F1-TAATAAGCTTCCGTTGAGGTTGTTGTC and R1-ATAGATCTGTTCCCTCCCTTGCGG, resulting in a 517 bp fragment containing the promoter and the first 2 bases of the coding sequence. The phoC promoter was cloned from cosmid SC4643A by PCR with the primer pair F2-TAATAAGCTTCCGCGTGCTCGCGTGTGA and R2-TGGGATCCATGTGCTCGTGTCGTA, resulting in a 532 bp fragment containing the promoter and the first 10 bases of the coding sequence. The phoD promoter was cloned from cosmid SC466 by PCR with the primer pair F3-TAATAAGCTTCCGCGTGCTCGCGTGTGA and R3-ATAGATCTGTTCCCTCCCTTGCGG, resulting in a 529 bp fragment containing the promoter and the first 98 bases of the coding sequence (a region important for expression of this gene; see Results). The different nucleotide distance from the fusion site to the translation start codon of the xylE gene for the phoD promoter as compared to those of phoA and phoC might affect the intensity of expression from the different promoters.

**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. lividans</em>  J1 1326</td>
<td>Wild-type</td>
<td>John Innes Centre*</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M145</td>
<td>Wild-type</td>
<td>Kieser et al. (2000)</td>
</tr>
<tr>
<td><em>S. coelicolor</em> INB101</td>
<td>M145 ΔphoP</td>
<td>Rodriguez-Garcia et al. (2007)</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>F′ φ80 lacZ ΔM15 Δ(lacZYA-argF) U16 recA1 endA1 hsdR17 (r− m−) supE44 med λ− thi-1 gyrA relA</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>Vector system for the cloning of PCR products</td>
<td>Promega</td>
</tr>
<tr>
<td>pGEM-PphaA</td>
<td>PCR product carrying the phoA promoter cloned into pGEM-T Easy</td>
<td>This work</td>
</tr>
<tr>
<td>pGEM-PphoC</td>
<td>PCR product carrying the phoC promoter clone into pGEM-T Easy</td>
<td>This work</td>
</tr>
<tr>
<td>pGEM-PphoD</td>
<td>PCR product carrying the phoD promoter cloned into pGEM-T Easy</td>
<td>This work</td>
</tr>
<tr>
<td>pGEM-PphoAp</td>
<td>pGEM-PphoA minus the MluI–BamHI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pGEM-PphoDp</td>
<td>pGEM-PphoD minus the MluI–MluI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pIJ4083</td>
<td>Promoter-probe plasmid using xylE as reporter</td>
<td>Clayton &amp; Bibb (1990)</td>
</tr>
<tr>
<td>pIJ4083-PphaA</td>
<td>phoA promoter cloned into pIJ4083</td>
<td>This work</td>
</tr>
<tr>
<td>pIJ4083-PphoC</td>
<td>phoC promoter cloned into pIJ4083</td>
<td>This work</td>
</tr>
<tr>
<td>pIJ4083-PphoD</td>
<td>phoD promoter cloned into pIJ4083</td>
<td>This work</td>
</tr>
</tbody>
</table>

*Collection of micro-organisms of the John Innes Centre, Colney Lane, Norwich NR4 7UH, UK.*
After PCR amplification the promoters were cloned into pGEM-T Easy (Promega). All the promoters were sequenced using pUC/M13 forward and reverse primers in a Perkin Elmer ABI PRISM 310 Genetic Analyzer, extracted from the plasmid via digestion with HindIII/BglII or HindIII/BamHI and ligated into pJ4083 in order to construct transcriptional fusions with the xylE reporter gene (Clayton & Bibb, 1990). The obtained promoter-probe plasmids pIJ4083-PhoA, pIJ4083-PhoC and pIJ4083-PhoD were first transformed into Streptomyces lividus, then extracted, confirmed by restriction with HindIII and XbaI and transformed into S. coelicolor.

**Promoter expression studies.** Promoter expression was studied using the xylE reporter gene of plasmid pIJ4083. Approximately 3 × 10⁷ spores were pregrown in 2 x TY medium (Kieser et al., 2000) for 8 h at 30 °C and 250 r.p.m. After centrifugation they were resuspended in 100 µl R5 medium (Kieser et al., 2000) and used to inoculate 100 ml R5 medium supplemented with high (1.8 mM) or low (0.009 mM KH₂PO₄) concentrations of added phosphate. Thiostrepton was used as selective antibiotic at a final concentration of 5 µg ml⁻¹. Samples were taken from two flasks at 18, 24, 27, 30, 33, 36, 39 and 48 h and catechol-2,3-dioxygenase activity was measured in triplicate as described by Kieser et al. (2000).

**DNA–protein binding assays.** To detect binding of PhoP to the promoters, studies were performed by EMSAs using glutathione S-transferase (GST)–PhoP and GST–PhoPBD (DNA-binding domain) fusion proteins as described by Sola-Landa et al. (2005). The promoters were excised from the pGEM-T Easy plasmid with restriction enzymes and labelled at both ends with digoxigenin using the DIG Oligonucleotide 3'–Labelling kit, 2nd generation (Roche Applied Science).

Samples were loaded onto a 5 % polyacrylamide native gel (29:1) in 0.5 % TBE buffer and electrophoresis was run for 5 h at 80 V. Afterwards the gel was electroblotted onto a nylon membrane for 1 h at 200 mA in 0.5 % TBE buffer and the DNA was fixed by UV cross-linking to the membrane. Labelled DNA was detected with antidigoxigenin antibodies by chemoluminiscence development with CDP-Star reagent (Roche Applied Science).

**Footprinting assays.** To obtain small promoter fragments, needed for footprinting studies, a fragment was deleted from the plasmids by digestion with BamHI/MluI in the case of pGEM-PphoA and with MluI alone in the case of pGEM-PphoD, using restriction sites located in the promoter and the flanking plasmid region but maintaining the annealing sequences for M13-20 and reverse primers. The plasmids obtained by religation were used to amplify the promoter fragments of phoA (PphoAp) and phoD (PphoDp) for DNAse I footprinting studies using M13-20 and reverse primers, one of them fluorescein-labelled. The assays were performed by the fluorescein labelling procedure as described by Rodriguez-Garcia et al. (1997) using the GST–PhoPBD protein as described by Sola-Landa et al. (2005). After amplification the PCR products were purified by agarose-gel electrophoresis and the DNA concentrations were determined by spectrophotometry with a GeneQuant spectrophotometer (Amersham Biosciences).

The reaction components were the same as in the DNA binding studies. Labelled DNA fragments (0.28 pmol) and GST–PhoPBD 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 and 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 × 10⁻⁴ units) at 25 °C for 1 min and stopped with 120 µl 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) and compared with the corresponding sequencing reaction using the same labelled primer as used for the amplification. Results were analysed with the Fragment Manager program (Amersham Biosciences).

**RNA extraction and primer extension.** The S. coelicolor M145 derivatives containing pIJ4083-PphoA, pIJ4083-PphoC and pIJ4083-PphoD were grown under the same conditions as described for the expression studies up to the time of maximum expression of each promoter (36 h for phoA and phoC; 39 h for phoD; see Results). RNA was then isolated by the hot phenol procedure adapted to Streptomyces (Patak et al., 2003). The transcriptional start sites were determined by primer extension using the 5'-fluorescein-labelled oligonucleotide O6-GCGATCGCTGCCACTGC as a primer (complementary to the 5' coding region of xylE from pIJ4083 vector). The reaction product was loaded in an ALF DNA sequencer (as described above) and analysed with the Fragment Manager program.

**RESULTS**

Three different S. coelicolor alkaline phosphatase genes: bioinformatic analysis indicates that PhoA, PhoC and PhoD are extracellular enzymes

Three putative alkaline phosphatase genes, phoA, phoC and phoD, were identified in the genome of S. coelicolor by a comparative search with the phoA gene of S. griseus (corresponding to the sequenced alkaline phosphate protein; Moura et al., 2001). The encoded proteins PhoA (558 aa, SCO2286), PhoC (553 aa, SCO0828) and PhoD (551 aa, SCO2068) (Fig. 1) showed amino acid identities of 73, 67 and 36 %, respectively with S. griseus PhoA. Bioinformatic analysis of the three proteins using the PFAM and SYSTERS programs revealed that all three contain an alkaline phosphate domain. The phoA- and phoC-encoded proteins showed high similarity to the alkaline phosphatases of Bacillus species, whereas phoD encodes a protein with high similarity (85 % identical residues) to a well-known Ca²⁺-dependent phospholipase D precursor of Streptomyces chromofuscus (Stiegelt et al., 2001; Yang & Roberts, 2002). This phospholipase catalyses two reactions: (i) hydrolysis of different phospholipids to generate phosphatidic acid, and (ii) transphosphatidylation with primary or secondary alcohols to generate different phospholipids. Therefore, S. coelicolor PhoD seems to correspond to a phospholipase D (see Discussion).

Since the total phosphate activity in S. coelicolor is a mixture of the different phosphatases, expression of each gene was quantified separately by coupling it to the xylE reporter gene.

Alkaline phosphatases and related proteins may be extracellular enzymes involved in phosphate scavenging or they may play roles in secondary metabolite biosynthesis (e.g. streptomyacin-6-phosphate phosphate encoded by strK (Mansouri & Piepersberg, 1991)) or in catabolic reactions (e.g. degradation of xenobiotics). Hydrophobicity plots using two different programs, TMpred and TMMHMM, predicted all three proteins PhoA, PhoC and PhoD to be soluble proteins.
PhoA (SCO2286) and PhoD (SCO2068) were shown to be secreted via the twin arginine translocation (Tat) pathway, a major route for protein secretion in *Streptomyces* (Widdick et al., 2006). Their N-terminal regions contain the conserved twin-arginine motif defined as R-R-X-W-W, where W represents a hydrophobic amino acid (Berks, 1996). Using the TatP algorithm (Bendtsen et al., 2005) for prediction of proteins exported via the Tat pathway we identified a clear Tat motif in PhoC, with the sequence GAAARHLGRRRFL TVTAA (amino acids 23–40), nearly identical to the Tat motif RAAARSLGRRRFL TVTGA (amino acids 27–44) predicted for PhoA (Widdick et al., 2006), suggesting that PhoC might be secreted via the Tat pathway.

Using neural networks and hidden Markov models trained on Gram-positive bacteria for protein secretion via the Sec or the SRP pathway, PhoA and PhoD are also predicted to be secreted. The cleavage site of the PhoA signal peptide is predicted to lie between amino acids 62 and 63 with a probability of 95.3%. The corresponding sequence is ASA/ A (／ indicates the cleavage site). The cleavage site of PhoD is located (with a probability of 81.3%) between amino acids 44 and 45 in the sequence ADAA/ A. The overall probability for both proteins to be secreted is 95.5% for PhoA and 100% for PhoD. The above-mentioned models, which are trained to predict proteins secreted via the Sec or the SRP pathway, indicate that PhoC is not secreted through these secretion pathways (with a secretion probability of only 30.5%).

Expression of *phoA* is strongly induced by phosphate limitation and this induction is PhoP-dependent

The promoter of *phoA* (*P*_{phoA}) in *S. coelicolor* was weakly expressed in high phosphate but highly expressed in low-phosphate conditions (Fig. 2a). In phosphate-limited batch cultures *phoA* is efficiently expressed, as measured by quantification of reporter activity, and reached its maximum activity at about 39 h (Fig. 2a), decreasing thereafter.

Expression of *phoA* was nearly non-existent in the *S. coelicolor* *ΔphoP* mutant, indicating that PhoP is strictly required for expression of this promoter, i.e. *phoA* expression is dependent upon activation by PhoP.

The promoter of *phoC* shows a weak transcriptional activity and is not PhoP-dependent

Expression of *phoC* in wild-type *S. coelicolor* M145 is much lower than that of *phoA* measured using the same reporter system. The profile of expression of *phoC* was clearly

---

**Fig. 1.** Physical map of the *S. coelicolor* DNA regions around *phoA*, *phoC* and *phoD* (black arrows). The arrows indicate ORFs and the orientation of transcription.
different from that of phoA. It was induced under low-phosphate concentrations, activity reaching its maximum after 36–39 h in R5 phosphate-limited medium, and not decreasing significantly until after 48 h of growth (Fig. 2b). However, its expression was not maintained after 30 h in phosphate-supplemented medium, indicating that this gene might be subject to a complex dual regulation by phosphate. This gene does not encode a typical alkaline phosphatase (see Discussion) and its expression may be modulated differently in low- and high-phosphate conditions.

The expression of phoC is negatively regulated by phosphate in both the wild-type and the ΔphoP mutant. In conditions of phosphate sufficiency the expression of phoC is only active in early growth (up to 30 h) then abruptly decreases. This early expression of phoC is not observed in the phoP mutant. A similar observation was made in the case of phoD. In order to explain these observations, we can argue that phoC and phoD are expressed during the exponential growth phase in high-phosphate conditions by a transcription factor that...
depends on the presence of PhoP. The later expression of phoC observed under phosphate-limited conditions is not under the positive control of PhoP, since its expression is even higher in the phoP mutant strain; this indicates the existence of an unknown phosphate-responsive regulator, probably detecting intracellular phosphate limitation, which is more severe in the case of the phoP mutant due to its inability to activate the expression of genes of the PHO regulon.

Expression of phoD is highly regulated by phosphate and partially PhoP-dependent

The expression of phoD increased under low-phosphate conditions, reaching an expression level nearly as high as that of phoA. Its expression was also significantly reduced by 1.8 mM phosphate.

In the ΔphoP mutant the overall phoD expression decreased significantly (but was not completely suppressed) when compared to wild-type strain M145, indicating that although PhoP is clearly involved in the phosphate control of phoD, its expression is not as strictly PhoP-dependent, as observed for phoA (Fig. 2c). In conditions of phosphate limitation, the expression of phoD is very active throughout growth and this expression is clearly dependent upon PhoP. In conditions of phosphate sufficiency, the expression of phoD is active in early growth then abruptly decreases as for phoC; this early expression is not detected in the ΔphoP mutant whereas a late expression (40 h) is observed. Our results suggest that another regulatory element besides PhoP plays a role in phoD induction under conditions of phosphate limitation.

Characterization of the promoters by primer extension

The transcription start points of the phoA, phoC and phoD genes were determined by primer extension. In all three cases the origins of transcription were identified and, once the +1 sites were known, the corresponding −10 and −35 boxes of each promoter were established by comparison to the matrices reported by Bourn & Babb (1995) for Streptomyces that take into account the nucleotides occurring in 13-nucleotide stretches, including the −10 or −35 consensus hexamers, as described by Sola-Landa et al. (2005).

Two different transcription start points (tsp1 and tsp2) were found upstream of the phoA open reading frame (Fig. 3). The first and stronger one was located at a guanosine 25 bp upstream of the start codon and corresponds to a −10 box (TCGAAT) and −35 box (CAAAACC) separated by 18 bp. For the second transcription start point, located 53 bp upstream of the start codon, no consensus −10 and −35 boxes could be identified, suggesting that this second transcription start point is probably recognized by a different sigma factor. A putative RBS with the consensus core sequence GGAGG was found 6 bp upstream of the ATG translation start initiation codon of phoA.

Transcription of phoC initiates from two different transcription start points (tsp1 and tsp2) located 25 bp and 50 bp upstream of the start codon (Fig. 4). For both sites possible −10 and −35 boxes with the sequences −10 TCCGGC/−35 TTTTGT (tsp1) and −10 CAGGGC/−35 ATCACT (tsp2) separated by 17 bp (tsp1) and 18 bp (tsp2) could be identified. A possible RBS with the core sequence AGAGG is located 7 bp upstream of the start codon of phoC.

Transcription of phoD initiates at the first guanosine of its GTG translation start codon, for which no RBS was identified. A second GTG codon is found 69 bp downstream, but comparison of PhoD with the homologous proteins from other Streptomyces sequences shows high conservation within the first 23 amino acids and as those include the Tat secretion signal predicted by Widdick et al. (2006), translation is likely to start at the first GTG (Fig. 5). Its −10 box (TAACCT) and −35 box (CCCCACT) are separated by 18 bp and overlap with the second protected region and the PHO box repeats located in it (Fig. 5).

All transcription initiation points were consistent with the identified −10 and −35 regions. We cannot exclude, however, other minor transcription start points that do not show up when using the primer extension technique.
Upstream of the phoD gene there are three ORFs transcribed in the same orientation (Fig. 1); one of them encodes a conserved hypothetical protein (SCO2065) and the other two encode putative membrane proteins (SCO2066/SCO2067). Downstream of phoD there is a conserved hypothetical protein arranged in the opposite orientation. In the expression studies we observed promoter activity for the DNA region upstream of phoD (Fig. 2). The possibility that phoD is additionally transcribed in an operon together with these three genes seems unlikely because of the 14 bp palindromic sequence located between the transcription start point of phoD and SCO2067 (Fig. 5) that could form a stable stem–loop structure in the RNA and function as a transcriptional terminator.

PhoP binds to the promoters of phoA and phoD, but not to that of phoC

The interaction of PhoP with the three promoters was tested by EMSAs using the S. coelicolor GST–PhoP fusion protein as well as a truncated version of PhoP containing only its DNA-binding domain (C-terminal region; GST–PhoPDBD). The PhoPDBD fragment shows constitutive binding to PHO boxes, independent of its phosphorylation status (Sola-Landa et al., 2005).

The promoters of phoA (Fig. 6a) and phoD (Fig. 6c) showed a clear shift of the labelled DNA band. In both cases the binding was stronger with the truncated PhoPDBD than with the complete PhoP protein. In the EMSAs performed with the phoD promoter up to four bands were observed, indicating that several DNA–protein complexes were formed due to binding of increasing amounts of PhoPDBD to the three Pho boxes existing in this promoter (see below), whereas the promoter of phoA formed only one shifted band.

An interesting finding was that the promoter of phoC did not show a shift reaction with either the complete PhoP or the truncated PhoPDBD version (Fig. 6b) under the same conditions as used for EMSA that showed mobility shifts for the phoA and phoD promoters. These results agree with the expression studies (Fig. 2b) and suggest that the phoC promoter lacks adequate PHO boxes. Computer analysis of the phoC promoter confirmed the absence of PHO boxes in this promoter, supporting the observed lack of binding of the PhoP response regulator to this DNA region.

Footprinting assays of phoA and phoD: identification of PHO boxes in these promoters

To locate the PHO box repeat units in the promoters of phoA and phoD, footprinting assays were performed using...
the PhoP<sub>DBD</sub> protein to protect them against DNAse I digestion.

Results of the footprinting studies identified in the phoA promoter a PhoP-protected region of 29 bp in the coding strand and of 29 bp in the complementary strand, overlapping by 23 bp. In this region one complete Pho box containing two repeats of 11 bp (Sola-Landa et al., 2005) with the sequence 

```
GTTCTCACGGT
GTTCAT-GACTC
```

was identified (the first nucleotide of each repeat is underlined) (Fig. 7). This sequence is located in the coding strand 103 or 75 bp upstream of the experimentally determined transcription start points tsp1 and tsp2, respectively (see below).

In the phoD promoter two protected regions of 38 bp and 28 bp (separated by 6 bp) in the coding strand and 33 bp and 27 bp (separated by 16 bp) in the complementary strand were identified, overlapping by 27 bp and 11 bp, respectively (Fig. 8).

The upstream protected region contains three direct 11 nt repeats located in the complementary strand with the sequence 

```
GTTCGCCCACT C GCGCG-TGTAACC
```

separated by one nucleotide (C) between the first and the following two repeats (Fig. 8) (see Discussion). The Pho box repeats in phoD are located 10 bp from the experimentally determined transcription start point, overlapping with the predicted –10 and –35 boxes (see below).

**DISCUSSION**

Three different alkaline phosphatase genes were initially described in the S. coelicolor genome (Moura et al., 2001; Bentley et al., 2002). Interestingly, similar BLAST searches in the genomes of Streptomyces avermitilis and Streptomyces scabies only identified two possible alkaline phosphatases, corresponding to phoA and phoD, so the phoC gene of S. coelicolor seems to be a more recent addition to the S. coelicolor genome and might be involved in the biosynthesis of some S. coelicolor-specific metabolite. PhoA of S. coelicolor corresponds to an authentic alkaline phosphatase with high similarity to the well-known extracellular alkaline phosphatase of S. griseus (Moura et al., 2001) and other micro-organisms. It has been shown to be secreted via the Tat pathway (Widdick et al., 2006) but also contains a typical leader peptide as described for Sec-dependent secretion (Schaerlaekens et al., 2004).

Expression of phoA is strongly activated by phosphate limitation (Fig. 2a) and is strictly dependent upon the binding of PhoP to the consensus PHO box identified in its promoter (see below). Transcriptional analysis revealed the existence of two transcription start sites, tsp1 and tsp2, allowing the definition of two promoters. DNAse I footprinting experiments revealed the presence of putative PHO boxes (two direct repeats of 11 bp) located 103 and 75 bp upstream of tsp1 and tsp2 respectively. These PHO boxes are in the
Phosphate limitation, the expression of not directly dependent upon PhoP. Under conditions of early PhoP-dependent phase up to 30 h and a later phase and phoD positions of an activator site for both promoters that are thus both likely to be regulated by phosphate availability.

phoC encodes an alkaline phosphatase similar to PhoA, but its regulation pattern is clearly different from that of phoA and phoD. Its expression can be divided into two phases: an early PhoP-dependent phase up to 30 h and a later phase not directly dependent upon PhoP. Under conditions of phosphate limitation, the expression of phoC is active in early growth stages but keeps on increasing during later growth and is even higher in the phoP mutant.

Although the TatP program (Bendtsen et al., 2005) predicts PhoC to be secreted via the Tat pathway, the KEGG database, a knowledge base for systematic analysis of gene functions (Kanehisa et al., 2006), classifies PhoC as a phosphoric monoester hydrolase that may be involved in two pathways: (i) $\gamma$-hexachlorocyclohexane degradation and (ii) folate biosynthesis. Although both pathways occur in S. coelicolor (PATHS SCO00361 and SCO00790, respectively) there is no unequivocal evidence to conclude that PhoC corresponds to the phosphatase catalysing the dephosphorylation steps of these pathways.

The lack of binding of the PhoP response regulator to the phoC promoter suggests that the observed phosphate regulation of the phoC gene is not mediated by direct interaction of PhoP, in agreement with the lack of PHO boxes in this promoter region. Phosphate regulation of phoC during the early growth phase might involve an intermediary regulator whose expression might be under the control of PhoR/PhoP. The phosphate-response regulator PhoP binds to a significant number of phosphate-regulated genes in Streptomyces (Sola-Landa et al., 2005), but other phosphate-controlled genes lack Pho boxes and are not recognized by PhoP (Rodriguez-Garcia et al., 2007).

The S. coelicolor phoD gene corresponds to a novel-type Ca$^{2+}$-dependent phospholipase similar to that of S. chromofuscus (85% identical amino acid residues). These phospholipases of S. coelicolor and S. chromofuscus show little homology with other members of the phospholipase D superfamily. This PhoD phospholipase is also different from classical phospholipases described in other Streptomyces species. The PhoD proteins of S. coelicolor and S. chromofuscus lack the identifiable HKD motif common to members of the phospholipase D family. The S. chromofuscus enzyme has been characterized biochemically and shows phosphodiesterase activity; it is secreted and cleaved by an extracellular protease, increasing its enzymic activity (Geng et al., 1999; Yang & Roberts, 2002). This enzyme binds to membrane phosphatidylcholine and facilitates vesicle aggregation and fusion (Stieglitz et al., 1999, 2001).

Transcriptional analysis revealed the existence of a unique transcriptional start point coinciding with the first base of the translation initiation codon, allowing the definition of an unique promoter. The expression of phoD is activated under phosphate-limited conditions by PhoP, which binds to the two regions of 11 bp repeat units (PHO box) found in its promoter, overlapping partially with its predicted −10 and −35 boxes. The formation of up to four DNA–PhoP bands in the EMSA experiments correlates with the distinct organization of the PHO boxes in the promoter of this gene. The activation of phoD in the mutant strain S. coelicolor ΔphoP (INB101) is weaker than that in the wild-type strain. This activation in the ΔphoP mutant under phosphate-limitation conditions suggests that another regulatory factor besides PhoP plays a role in phosphate control.

The sequence of PHO boxes protected by binding of the PhoP protein was established recently in the promoters of the pstS, phoRP and phoU genes (Sola-Landa et al., 2005; Rodriguez-Garcia et al., 2007). However, the number of validated PHO boxes in Streptomyces genomes is still very limited. The presence of two 11 bp direct repeats in the phoA promoter and six repeats in the phoD promoter that match the protected region allowed us to establish a new, better-fitting sequence for the Pho box as G(82%)G/T(85%)T(77%)C(90%)A(59%)Y(82%)Y(74%)Y(72%)R(62%)Y(64%)Y(74%).

Duplication of the number of PHO boxes in certain promoters is related to a potential modulation of the strong phosphate control (Mendes et al., 2007). The
formation of up to four DNA–PhoP protein complexes in the binding reaction of the phoD promoter suggests that this promoter is subject to different degrees of PhoP control depending on the level of the phosphorylated response regulator in the cell.

**ACKNOWLEDGEMENTS**

This work was supported by grants of the CICYT (BIO2003-01489), Madrid (Spain) and the European Union (Project ACTINOGEN, LSHM-CT-2004-005224) Brussels. A. K. Apel received a F.P.U. fellowship of the Ministry of Education and Science. We thank J. Merino, B. Martín, B. Aguado and A. Casenave for their excellent technical assistance.

**REFERENCES**


Phosphate control in *Streptomyces coelicolor*


Edited by: J. Anné