

Target genes and structure of the direct repeats in the DNA-binding sequences of the response regulator PhoP in *Streptomyces coelicolor*

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ABSTRACT

Expression of genes belonging to the *pho* regulon in *Streptomyces coelicolor* is positively regulated (as shown by comparing the wild-type and a Δ *phoP* mutant) by binding of the response regulator PhoP to 11-nt direct repeats (DRUs). These sequences have been found in over 100 genes of *Streptomyces coelicolor*; 20 of them were cloned and the binding of PhoP^{DBD} to most of their promoters has been shown by electrophoretic mobility shift assays. Deletion experiments showed that at least two DRUs are required for proper binding of PhoP^{DBD}. Deletion of 1 nt leaving a 10-nt direct repeat reduced drastically binding of PhoP^{DBD}. Three different types of operators have been identified. Complex operators (class III) contain up to six DRUs, some of them with poor conservation of the 11-nt consensus sequence, which however were protected by PhoP^{DBD} in footprinting analyses. A cooperative binding of PhoP^{DBD} molecules initiated at conserved core DRUs appears to be the mechanism involved in binding of several PhoP^{DBD} monomers to those complex operators. The information theory-based model that incorporates the positive or negative contribution to the binding of PhoP^{DBD} of adjacent sequences has been used to deduce the structure of PHO boxes and the relevance of each DRU.

INTRODUCTION

Streptomyces species and other actinomycetes are soil-dwelling Gram-positive bacteria that produce an impressive array of secondary metabolites (1), many of which possess important pharmacological activities (2,3).

The ability to produce this variety of secondary metabolites is due to a wealth of genes, usually arranged as clusters (4,5) that are being found in the sequenced genomes of *Streptomyces* species (6,7).

Most of the secondary metabolites are secreted (8) and serve as inter-cellular talk signals in pure culture (e.g. quorum sensing) (9,10) or as inter-generic weapons or communication signals (e.g. anti-bacterial, anti-fungal, plant-growth promoting substances) (11,12). The metabolism of *Streptomyces* species in soil and also in pure cultures is strictly regulated by the availability of nutrients, particularly inorganic phosphate (13,14).

Defined sets of genes that show specific overexpression patterns are triggered in response to phosphate starvation (15). Expression of genes encoding secondary metabolites and, therefore, production of these compounds takes place only under phosphate-limiting conditions (16,17), whereas high phosphate conditions favour expression of house-keeping genes, particularly those related to nucleic acid and protein synthesis. Silent gene clusters that are not significantly expressed may be triggered if phosphate-controlled mechanisms are disrupted, thus providing an increasing arsenal of rare secondary metabolites with potentially novel biological activities. Not all secondary metabolite genes are equally sensitive to phosphate control. Polyketide and aminoglycoside compounds are more sensitive to phosphate regulation than non-ribosomal peptides (14,18,19) and this is likely to be due to the presence of specific control sequences in their promoter regions and in the genes-encoding enzymes providing precursors for these metabolites (20).

The control of phosphate-regulated genes in *Streptomyces coelicolor* and *Streptomyces lividans* is mediated by the two-component system PhoR-PhoP (20–22). Recently, we purified the regulatory fused proteins GST-PhoP and GST-PhoP^{DBD} (a truncated version with the DNA-binding domain) and showed that they bind to PHO

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boxes, close to the -35 sequence in the *pstS* and *phoU* promoters. Each of these PHO boxes is formed by direct repeat units (DRu) with the $G_{G/T}TCAYYYR_{G/C}G$ consensus sequence (23).

The identification of PHO boxes was based on the footprinting analysis of three phosphate-regulated genes. However, a computer search of putative phosphate-controlled genes showed a degree of variability in some of the nucleotide positions of the DRus. The divergence with respect to the consensus sequence in the PHO boxes may affect the binding of phosphorylated PhoP and, therefore, the degree of phosphate control.

Moreover, there are usually two or four DRus in the PHO boxes of phosphate-regulated genes, although the presence of up to eight DRus has been reported in the upstream region of the *phoRP* cluster of *Streptomyces natalensis* (19). In early studies in *Escherichia coli* (24), the presence of an odd number (e.g. seven DRus in the upstream region of the *ugp* operon) was proposed. It is unclear, however, whether a single DRu will allow formation of stable PhoP–DNA complexes. It is likely that the presence of four or more adjacent DRus in a region may allow binding of phosphorylated PhoP to an otherwise poorly conserved DRu sequence. It was, therefore, of great interest to study the binding of PhoP to a significant number of native putative PHO boxes and also to engineer new promoters with modified DRus in order to establish precisely the optimal nucleotide sequence and the number of DRus required for stringent or ‘relaxed’ phosphate control of gene expression.

In this study, we analyse the importance of the number of DRus for binding of PhoP^{DBD} and the presence of PHO boxes in different phosphate-regulated promoters. We have modified the nucleotide sequence of the PHO boxes or the distance between DRus by directed mutagenesis and this has allowed us to define a model for the binding of PhoP to those boxes.

MATERIALS AND METHODS

DNA manipulations

All the oligonucleotides used in this work are listed in Table S1 (Supplementary Data). Promoters with putative PHO boxes were amplified by PCR and the products were cloned into pBluescript KS+ plasmid (Stratagene). The correct amplification was tested by sequencing using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). For analysis of *pstS* promoter, two new restriction targets were created with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) using plasmid pGEM-P*pstS* (23) as template. The PstS1/PstS2 oligonucleotide pair was used for introducing an Acc65I site, and the PstS3/PstS4 for an SpeI site. The resulting plasmid was digested with these enzymes and the PHO boxes in the *pstS* promoter region were replaced by new sequences obtained by oligonucleotide pairing. For this, a mixture of 25 nM of oligonucleotides PstS5 and PstS6 or PstS7 and PstS8 was heated to 95°C for 5 min and cooled to 25°C with a cooling slope of 0.02°C/s. The final product was ligated with the vector plasmid (from which the original

PHO box had been removed) and transformed into *E. coli* DH5 α . The correct insertion was established by sequencing. Likewise, for modification of SCO1854, SCO1993 and *pstS* promoters the QuickChange system was used with oligonucleotide pairs PHO21/PHO22, PHO23/PHO24 and PstS9/PstS10, respectively.

Bioinformatic analysis of PhoP operators

The individual information theory programs makebk, delila, encode, rseq, dalvec, makelogo, ri, scan, multiscan and lister (25,26) were used to identify and analyse putative target sequences of the response regulator PhoP. The search for new operator sequences was carried out as described previously (15), using an individual information weight matrix constructed with the alignment of 18 DRus (model 0). These DRus stem from the experimentally validated operators of *pstS* (two DRus), *phoU-phoR* (four), *phoA* (two), *phoD* (six) and *pitH2* (four) [(23,28), Santos-Beneit, F., unpublished data]. Using the newly validated DRus, we constructed model I that comprises 37 DRus that form the core of the binding sites referred in Table 1. Model I was used to evaluate the DRus found in the protected regions, and to generate the logo and the walkers (25,29).

Promoter activity

The activity of promoters in *S. coelicolor* M145 and *S. coelicolor* Δ *phoP* (15) strains was measured using the reporter *xylE* gene in pIJ4083 plasmid, coding for a catechol-2,3-dioxygenase (30). Strains were grown in R5 medium with 40 μ M phosphate and samples were taken at different times, as described previously (23).

EMSA and footprinting analysis

DNA–PhoP^{DBD} interaction was analysed by the electrophoretic mobility shift assays (EMSA). The promoters were excised from the pBluescript plasmid by digestion with restriction endonucleases and labelled at both ends with digoxigenin with DIG Oligonucleotide 3'-End Labeling Kit, 2nd Generation (Roche Applied Science). The conditions for DNA–protein binding and detection were described previously (23). DNase I footprinting assays were performed by the fluorescent-labelling procedure (31), using the GST-PhoP^{DBD} protein (1.06 μ M) as described by Sola-Landa *et al.* (23).

RESULTS

Expression of phosphate-controlled *phoU*, *pstS* and *phoRP* genes requires PhoP: positive regulation

The availability of a deletion Δ *phoP* mutant of *S. coelicolor* (15) allowed us to compare the expression of three phosphate-regulated genes *phoU*, *pstS* and the *phoRP* cluster in the parental strain *S. coelicolor* M145 and the mutant Δ *phoP* under identical phosphate-limited conditions (for optimal expression of those genes). Expression of these three genes, as shown by time-course quantifications of the catechol oxygenase reporter activity (Figure 1), did not occur in the Δ *phoP* mutant under

Table 1. Promoters recognized by PhoP

Promoter region	Regulated gene and function	Operator structure ^a	Number of EMSA complexes	Strand	Distance to ATG	Distance to +1	Profile ^b	P-value ^c	PhoP regulation ^d
A. Class I: operators with 2 well-conserved DRu									
SCO2286	<i>phoA</i> , secreted alkaline phosphatase	<u>CC</u>	1 ^e	Coding	128	103	AA	>0.25	Activation ^e
SCO3790-1	SCO3790, putative phosphatase	<u>CC</u>	1	Coding	79	N.D.	0D	0.165	Activation
SCO4142	<i>pstS</i> , Pst transporter, phosphate-binding component	(EE) <u>CC</u>	1 ^f	Coding	114	39	A0	<0.000	Activation ^f
SCO6169-70	SCO6169, possible regulatory protein	<u>CC</u>	1	Coding	195	N.D.	00	>0.25	N.D.
SCO6169-70	SCO6170, putative oxidoreductase	<u>CC</u>	1	Non-coding	101	N.D.	00	>0.25	N.D.
B. Class II: operators with 3 conserved DRu									
SCO0033-4	SCO0034, unknown	<u>CCC</u>	1 ^g	Non-coding	127	N.D.	0D	0.034	Activation
SCO1906	SCO1906, putative phosphatase	<u>CCC</u>	1	Coding	92	N.D.	00	> 0.25	N.D.
SCO4261-3 ^h	SCO4261, possible response regulator	<u>CCC</u>	1 ^g	Non-coding	43	N.D.	aA	0.088	Repression
C. Class III: operators of complex structure									
SCO1393-4	SCO1394, possible glycosyl hydrolase	(E) <u>CC</u>	2	Non-coding	30	N.D.	A0	0.049	Activation
SCO2262-3	SCO2262, possible oxidoreductase	<u>ECC</u>	2	Coding	70	N.D.	0A	0.013	Repression
SCO2465-6	<i>hrdA</i> , principal sigma factor paralogue	<u>ECC</u>	2	Coding	80	N.D.	0A	0.052	Repression
SCO5447	SCO5447, putative metalloprotease	<u>ECCE</u>	3	Non-coding	214	N.D.	A0	0.26	Activation
SCO7697	SCO7697, possible secreted phytase	<u>CCE(E)</u>	3 ^g	Coding	122	N.D.	A0	0.001	Activation
SCO4228-9	<i>phoU</i> , regulator of the PHO response	<u>CCE_UE_S</u>	2 ^f	Coding	109	38	A0	0.143	Activation ^f
SCO4228-9	<i>phoRP</i> , PHO two-component system	<u>CCE_UE_S</u>	2 ^f	Non-coding	63	65	A0	<0.000	Activation ^f
SCO2878	SCO2878, unknown	<u>CCCE_UE_S</u>	2-3	Coding	42	N.D.	AD	<0.000	Activation
SCO1196	SCO1196, putative secreted protein	<u>CCC[2]E_S</u>	2	Non-coding	83	N.D.	A0	<0.000	Activation
SCO1845	<i>pitH2</i> , phosphate transporter	E _S [1]E _S E _U <u>CC[2]E_S</u> ⁱ	4 ⁱ	Coding	48	3	00	>0.25	Activation/repression ⁱ
SCO2068	<i>phoD</i> , phospholipase	<u>CCC[8]</u>	4 ^e	Non-coding	10	10	00	>0.25	Activation/repression ^e
		E _S [1]EE ^e		Coding					
D. Unclassified operators									
SCO4878-9	SCO4878, putative glycosyltransferase	4 DRu ^j	1 ^g	Coding	40	N.D.	00	>0.25	N.D.
SCO4878-9	SCO4879, hypothetical protein	5 DRu ^j	1	Coding	30	N.D.	A0	0.001	Activation

^aEach letter symbolizes a DRu and denotes the functional class. Class 'C' comprises DRus that form the core of the operator; 'E' comes from 'extension', 'E_U' from 'extension unstable' and 'E_S' from 'extension support'; see text for a complete explanation. The DRus that originated the information matrix are represented by underlined letters. In two operators, brackets enclose possible DRus that were not protected in the footprinting assays, but help to explain the number of EMSA complexes. Numbers in square brackets are the separation in base pairs between contiguous DRus.

^bTranscription profiles of the response to phosphate limitation. The first letter summarizes the transcription response of the *S. coelicolor* wild-type strain, and the second, the response of the Δ *phoP* mutant. 'A' means augmented expression, 'D' means decreased expression and '0', no detected change ($P > 0.1$). Data obtained from Rodríguez-García *et al.* (15).

^cThese *P*-values refer to the interaction contrast (or contrast 5; see Rodríguez-García *et al.* (15) for details). This comparison quantifies the differences between the response to phosphate limitation of the *S. coelicolor* wild type and that of the mutant Δ *phoP* strain. Low *P*-values support the regulatory role of PhoP.

^dThe type of the transcriptional control was inferred from the transcription profile or from the reference provided. In the cases of bidirectional promoter regions, which genes are controlled was decided from the transcriptomic results; exceptionally, there is no data for SCO2263, because no probe was present in the microarrays used.

^eAccording to Apel *et al.* (28).

^fAccording to Sola-Landa *et al.* (23).

^gAccording to Rodríguez-García *et al.* (15).

^hThe ORF SCO4262 was annotated as doubtful (6). Since it lies overlapping the putative promoter region of SCO4261, we considered that this ORF is not real.

ⁱAccording to Santos-Beneit, F. unpublished data.

^jThe fragment that contains both operators of the SCO4878–SCO4879 intergenic region, showed a unique complex in EMSA experiments. Since it is not possible to dissect the contribution of each operator to the mobility shift, only the number of DRus is indicated.

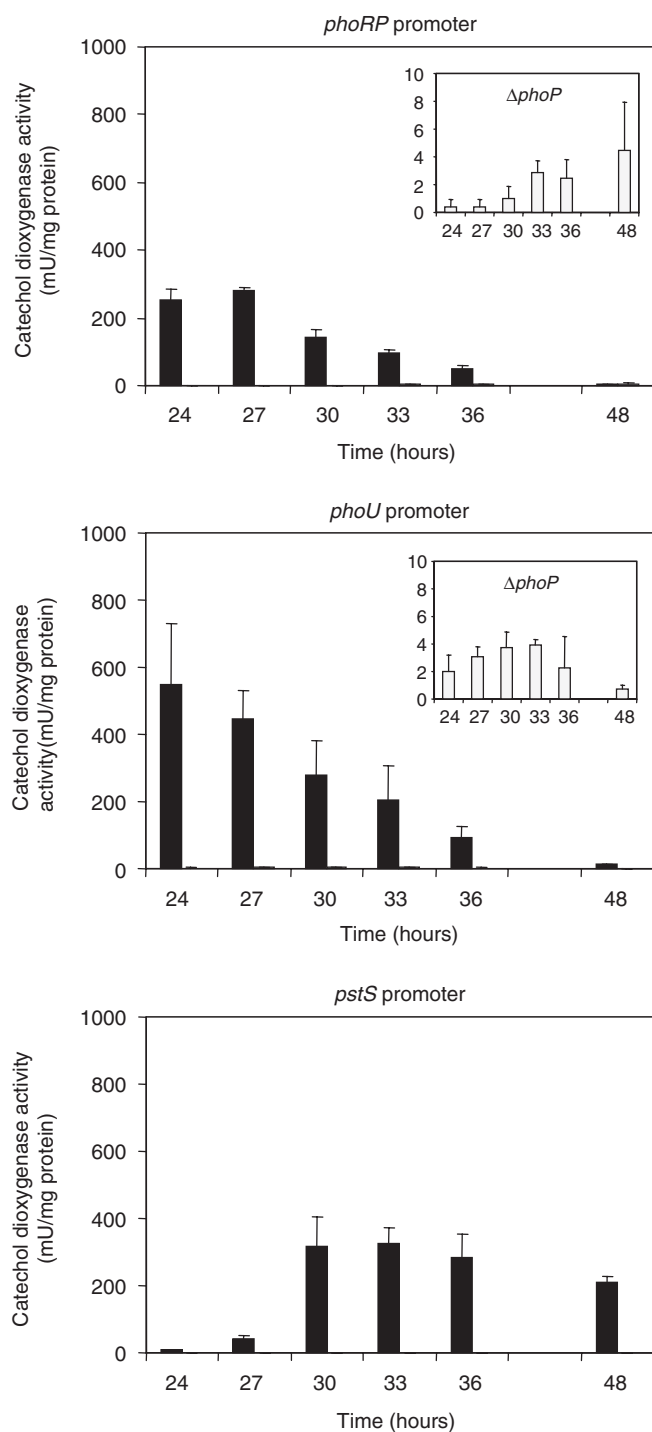


Figure 1. Promoter activity in *S. coelicolor* M145 (dark bars) and $\Delta phoP$ mutant (open bars) of *phoRP*, *phoU* and *pstS* using the *xyIE* gene (encoding a catechol dioxygenase) as reporter, growing in R5 liquid medium with 40 μ M phosphate. The activity in $\Delta phoP$ mutant is also represented in a larger scale (insets) for easier visualization. Vertical lines on top of each bar indicate SD values. No activity was detected with *pstS* promoter in the $\Delta phoP$ mutant.

conditions favouring gene expression. Only traces of expression of *phoRP* and *phoU* were found in the $\Delta phoP$ mutant (Figure 1, insets) and no expression at all was observed for *pstS*. These results prove that the

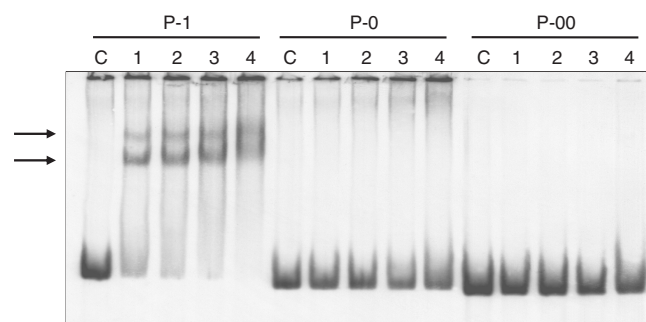


Figure 2. Analysis by EMSA of P-1 (native *pstS* promoter), P-0 and P-00 promoters (with deletions of one or two DRUs, respectively) using increasing concentrations of GST-PhoP^{DBD} protein. Lane C, control without protein; 1, 2, 3 and 4 reactions with 3.12, 6.25, 12.5 and 25 pmol of protein, respectively. The two shift bands of the *pstS* P-1 promoter are indicated by arrows. Note that there is no band in the gels with P-0 or P-00 promoters.

PhoP-mediated control of these genes is exerted by a positive mechanism, i.e. binding of PhoP to the PHO boxes (23) is strictly required for expression of these genes.

Two DRUs are required for PhoP binding

Using the native *pstS* promoter as model (containing two DRUs), we constructed different tailored promoters by directed mutagenesis. In a first step, we introduced two restriction targets, for the endonucleases Acc65I and SpeI, obtaining the native P-1 promoter as a portable cassette. Later, we replaced the PHO box by new sequences with only one DRU (P-0 promoter) or without any (P-00 promoter), preserving the -35 sequence. When binding of PhoP^{DBD} to these promoters was analysed by EMSA, results showed that both DRU are necessary for PhoP^{DBD} binding (Figure 2). While there is a clear binding of PhoP^{DBD} to the native promoter (forming two complexes with different mobility), no binding was observed in the P-0 (one DRU) or in the P-00 promoter (without any DRU) under the same reaction conditions.

Selection of candidate target promoters for PhoP regulation

Using an informatic matrix made with the sequence of PHO boxes of *pstS*, *phoRP-phoU* (23), *phoA*, *phoD* (28) and *pitH2* (Santos-Beneit, F., unpublished data) promoters, we analysed the genome of *S. coelicolor*, as indicated in Materials and Methods section. Several previously uncharacterized genes were found to possess sequences related to the proposed PHO box in their promoter region. Inspection of the candidate target sequences, analysis of the microarray data (15) for signs of PhoP-dependent regulation and concordance of the predicted gene functions with a possible role in phosphate metabolism were considered to choose candidates for experimental analysis. Twenty promoters were amplified by PCR and analysed by EMSA (see Figure 3 for those promoters showing positive binding). These include, in addition to promoters already studied (23,28), SCO0254 (encoding a hypothetical protein), SCO0908 (an unknown protein), SCO1196 (a putative secreted protein), SCO1356 (a putative iron-sulphur protein), SCO1394 (a possible glycosyl hydrolase),

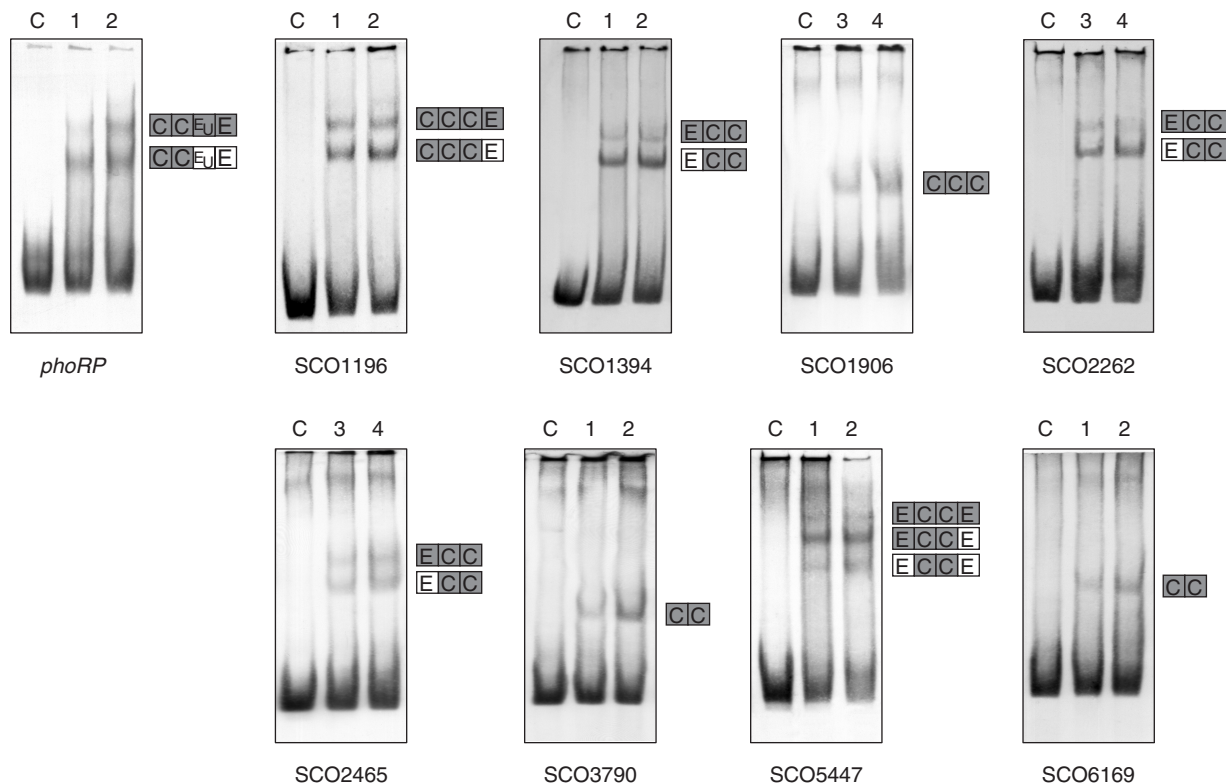


Figure 3. Analysis by EMSA of the binding of GST-PhoP^{DBD} to several promoters studied in the present work. Lane C, control without protein; 1, 3.12 pmol of protein; 2, 6.25 pmol; 3, 12.5 pmol; 4, 25 pmol of protein. The *phoRP* promoter was used as control. Only promoters giving positive shifts are shown. Promoter names are indicated under the picture. In the right side of every shift, a scheme is represented explaining the probable DRUs structure in the bands detected. C, E and E_U correspond with the different types of DRU, Core, Extension and Unstable (see text for explanation). Boxes presumably bound to PhoP are in grey and free boxes (not bound to PhoP) are in white.

SCO1854 (a possible integral membrane protein), SCO1906 and SCO3790 (putative phosphatases different from *phoA*, *phoC*, *phoD*), SCO1993 (an unknown protein), SCO2262 (a possible oxidoreductase), SCO2620 (a probable cell division trigger factor), SCO4451 (a probable export protein), SCO5447 (a putative neutral zinc metalloprotease), SCO6169 (a possible regulatory protein), SCO6722 (a putative regulator), *dnaB* (SCO3911), encoding a probable replicative DNA helicase, *hrcA* (SCO2555), encoding a heat-inducible transcriptional repressor, *hrdA* (SCO2465), encoding a RNA polymerase principal sigma factor, *frr* (SCO5627), encoding a ribosome recycling factor, *redQ* (SCO5887) and *redP* (SCO5888); these last two genes are involved in undecylprodigiosin (red) biosynthesis and constitute an example of secondary metabolite biosynthesis genes that might be directly (or indirectly) regulated by PhoP.

Relevance of nucleotides at positions 1 and 2 of a DRU: changing unmatching nucleotides to the consensus sequence restores PhoP^{DBD} binding

Considerable differences in PhoP^{DBD} affinity with these sequences were observed and in some of them no clear binding (as evidenced by EMSA) was detected after repeated gel shift experiments. In some cases, we observed a lack of binding even with promoters having DRUs

sequences relatively well conserved, but differing in specific nucleotides from the 11-nt consensus.

Two of these promoters (SCO1854 and SCO1993) were directly mutated and not matching nucleotides were replaced by the consensus ones (an A to a T in the second position and a CC to GT in the first and second position, respectively; Figure 4A). In both cases, the replacement resulted in a positive binding, confirming that the lack of binding was due to these specific nucleotides. The T in the second position of the direct repeat of SCO1854 and GT in the positions 1 and 2 (they were CC in the native promoter of SCO1993) proved to be essential for DNA binding of PhoP^{DBD} to those direct repeats.

DRUs of 10 nt and 11 nt

Each DRU was proposed to be 11-nt long. This fits well with the known number of nucleotides per turn of the double helix in the B-DNA (10.6 bp per turn) (helical pitch). However, the Z-DNA structure contains 10-nt per helix turn. We studied the functionality of operators consisting of one DRU of only 10 nt and another of 11 nt. A nucleotide belonging to the low-conserved region (position 10) of the first DRU of the *pstS* operator was deleted and PhoP^{DBD} binding was compared with that of wild-type promoter by EMSA (Figure 4B). Results showed that PhoP^{DBD} is able to bind the shorter 10-nt sequence, but with a very low efficiency, supporting the

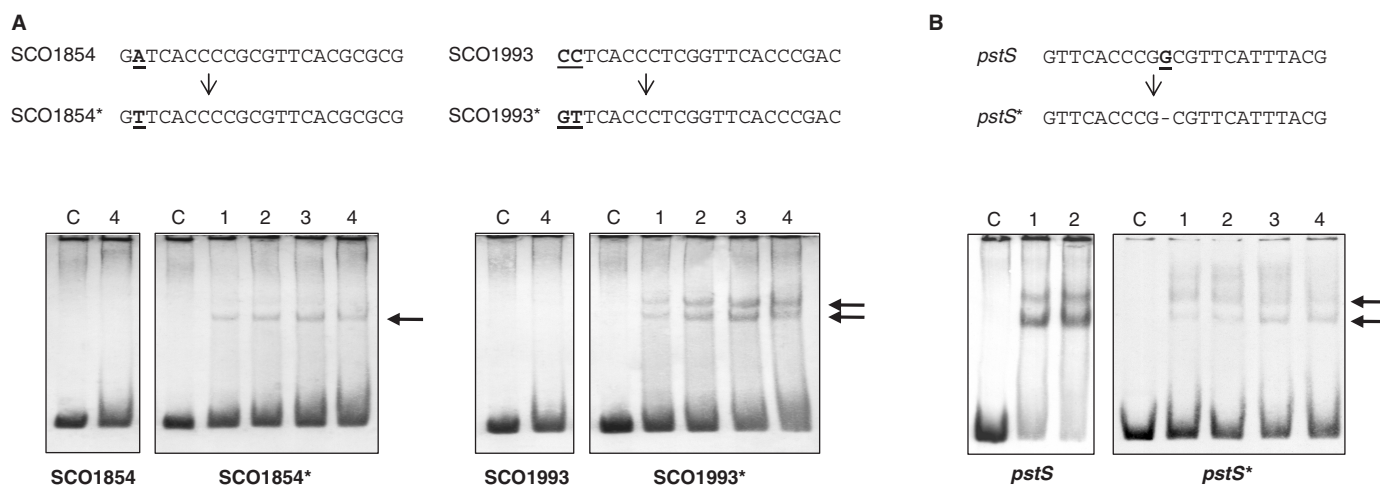


Figure 4. (A) Analysis of binding of PhoP^{DBD} to the SCO1854 and SCO1993 promoters. The native promoters were mutated (top) and the unmatching bases were replaced by consensus ones. Changed bases are shown in boldface and underlined. The new promoters are named with the same SCO number and an asterisk. Lower panels, EMSA using GST-PhoP^{DBD} protein. Lane C, control without protein; 1, 3.12 pmol of protein; 2, 6.25 pmol; 3, 12.5 pmol; 4, 25 pmol of protein. Note that at least a shift band (indicated by arrows) can be seen in mutated promoters, even with the lowest protein concentration (lane 1), whereas no band is detected in the wild-type promoters with the highest concentration (lane 4). (B) Analysis of the native *pstS* promoter and a mutant promoter containing a 10-nt DRU. The base deleted between the two DRUs is shown in boldface and underlined (top). The new promoter is named *pstS**. Note the drastic reduction in binding to the mutant promoter.

model of a DRU with 11 nt, although the number of nucleotides protected by PhoP^{DBD} must take into account the bending of the promoter DNA.

Footprinting identification and structure of the PhoP-binding sites

Because some DRUs are poorly conserved, promoters with positive binding were analysed by footprinting to know the exact number of DRUs and their organization in every one. In this study, we also included promoters of SCO0034 (encoding an unknown protein), SCO2878 (unknown protein), SCO4261 (a possible response regulator), SCO4878-SCO4879 (putative glycosyltransferase; hypothetical protein, respectively) and SCO7697 (a putative phytase) genes, that have been shown to be recognized by PhoP^{DBD} (15). The nucleotide sequences protected from DNase digestion by PhoP^{DBD} are shown for each promoter in Figure S1 (Supplementary Data).

We searched for DRUs in the protected regions using the information theory and programs (see Materials and Methods section). The results of this search led to the discovery of a complex structure of the PhoP^{DBD}-binding sites. Thus, the number of DRUs varied from a minimum of two to a maximum of five tandemly arranged DRUs in SCO2878 and in SCO4878-9 promoter regions. As occurs in the *phoRP* and *phoD* operators, several protected regions contained DRUs poorly conserved. Finally, in the SCO1196 protected region, a well-conserved DRU is 2-nt apart from three consecutive DRUs (Figure S1).

The analysis of the DRU organization together with the EMSA results allowed us to classify the promoters in three different types. The first class includes promoters with only two DRUs, like *pstS* (23), *phoA* (28), SCO3790 and SCO6169. This is the simplest structure and the repeats are well conserved (Table 1, panel A). In EMSA analysis

of those promoters, generally a unique band was detected; the exception of P-1 promoter will be discussed later. The second class is formed by operators with three conserved DRUs (Table 1, panel B). As occurs with the first type of promoters, only one complex was detected in EMSA. Therefore, both these classes of promoters can be defined as composed of DRUs that form a firm binding site: each DRU is occupied by a PhoP^{DBD} monomer and the full site is bound at the same time. In contrast, the promoters of the third class showed a complex DNA-protein stoichiometry.

Based on our findings, it is proposed that two (e.g. *phoD*) or three (e.g. SCO1196) conserved DRUs form the core of the binding site and are bound by PhoP^{DBD} at the first stage (denoted by C letters, for core, in Table 1, panel C). At higher protein concentrations, the length of bound sequence is extended to adjacent DRUs. This conclusion is supported by the information content analysis (see below). A PhoP cooperative oligomerization on the DNA has been proven for the *Bacillus subtilis* and *E. coli* PhoP orthologues (see Discussion section).

Information content analysis of the nucleotide sequences

The initial information-based model of the binding site (model 0) was refined taking into account the newly discovered DRUs. This model (model I, see Materials and Methods section) was built from 37 DRUs that form the core of the selected binding sites (Table 1). The sequence logo (25) that depicts the binding site is shown in Figure 5. The conservation of the first seven positions is clear and remarks the importance of these bases to establish specific protein-DNA contacts, whereas the last four positions contribute with little information. A similar pattern of conservation is found in the repeats that form the PHO boxes in other bacteria (see Discussion section).

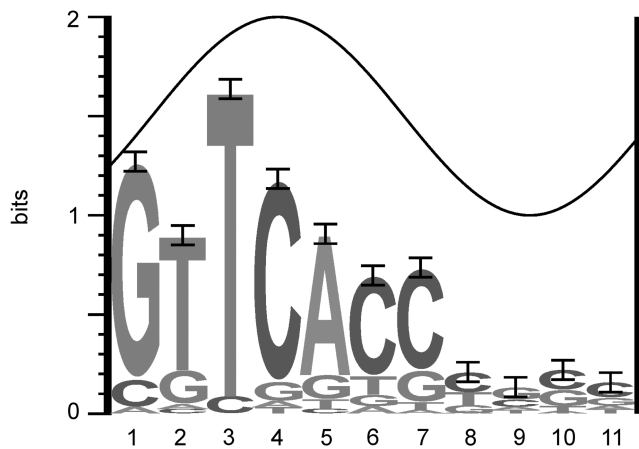


Figure 5. Sequence logo of the direct repeats of 11 nt that form the PhoP-binding-site unit. This logo corresponds to model I that comprises 37-DRUs of *Streptomyces coelicolor* (see Materials and Methods section). The height of each letter is proportional to the frequency of the base, and the height of the letter stack is the conservation in bits at that position (25). Note the high conservation of the first seven positions of the DRU. Error bars are shown at the top of the stacks. The total information (R_{sequence}) for the DRU is 8.08 ± 0.61 bits. The sine wave represents the accessibility of a face of the DNA (B-form, 10.6 bases of helical pitch) with the major groove centred at position 4. C in position 1 or an A or a C at position 2 have a clear negative effect on PhoP binding.

As proteins usually bind DNA from one face, it is expected that binding-site positions with conservation higher than 1 bit are located at the major groove (26). Thus, it is likely that PhoP^{DBD} contacts bases 1, 3 and 4 through the major groove of the DNA (Figure 5). A similar result has been obtained in *E. coli*, where the third and fourth bases are directly contacted by PhoB (32).

The sequence walkers method displays, for individual sequences, the contribution of each base to the conservation of the binding site (29). The walkers, as well as the individual information contents of the DRUs, or R_i (27), allowed us to analyse the requirements of PhoP^{DBD} for DNA recognition. Class I operators are composed of two DRU; i.e. one PHO box. As shown above, this is the simplest operator structure. The sequences of these sites are well conserved, as reflected by high R_i values and by the fact that the sequence GTTCAY is present in seven out of eight DRUs (the exception is the first DRU of *phoA*, Figure 6A). In the cases of class II operators, at least one of the three DRUs is well conserved ($R_i > 8$ bits; this is the $R_{\text{sequences}}$, Figure 5). The complex class III operators can be seen as composed of a core of two or three DRUs associated with other DRUs that are bound at higher PhoP^{DBD} concentrations. Those core DRUs show the same features than the DRUs of class I and II operators (all designated 'C' in Table 1). These features are: (i) the sites are made up with at least one DRU with $R_i > 8$ bits, except *phoA*; (ii) the DRUs are consecutive (null separator); (iii) the information content of the full sites (sum of individual R_i values) have a mean of 18.1 ± 1.29 bits (standard error) for pairs, and 19.5 ± 2.11 bits for trios; i.e. both sets have similar information content.

Nine of the fragments that were initially amplified for EMSA tests failed to contain true binding sites despite of showing certain sequence conservation. The comparison of walkers from true and false sites provides new rules to evaluate potential new sites and highlights the importance of key positions. Firstly, an adenine base at position 2 (A_2) seems to impede the binding. This is demonstrated, at least for two-DRUs sites, with the mutagenesis of the SCO1854 fragment (Figure 4). The replacement of an A for a consensus T makes the sequence capable of binding PhoP^{DBD}. This would also explain the lack of binding of SCO1356 and *tig* sites (Figure 6B). Secondly, two-DRUs sites appear to require a high conservation at the first six positions in both repeats. This is the case of C_1C_2 and C_1G_6 on the first DRU of SCO1993 and *hrcA*, respectively. Indeed, in three-DRUs sites, always the first and third DRU contain a G; a C_1 is found only in middle DRUs. This observation explains the lack of binding to the *redQ* sequence (Figure 6B). This sequence and that of *redP* contain three DRUs. The most remarkable features are the lack of conservation in the first positions and the presence of G_6 in every DRU. Finally, the sequences of SCO6722, *frr* and *tig*, despite of containing several potential DRU, lack core sites. That is, they lack two or three DRU-like sequences that accomplish the features of operator cores.

DISCUSSION

Different pathways known to be regulated by inorganic phosphate availability respond distinctly to different phosphate concentrations (20). Some genes are induced when the concentration of phosphate in the growth medium falls below 5 mM; others, when the concentration falls below 1 mM or less. For instance, the expression of the *pstS* gene is strongly induced when the concentration of phosphate falls below 0.1 mM (15). Although other regulators could also be involved in the control of the expression of a given gene, its different sensitivity to phosphate availability seems to rely on the affinity of the response regulator PhoP for its cognate-binding sequences (PHO boxes). These were identified as tandem reiterations of direct repeats of 11 nt with the consensus sequence $G_{G/T}TCAYYYR_{G/C}G$ where Y is a pyrimidine and R is a purine (23). The truncated version of PhoP was used because it has been shown previously that PhoP^{DBD} binds more efficiently to promoters than PhoP (23,28). In addition, it has also been shown in other microorganisms that the DBD domain binds in a 'constitutive' manner similar to the binding of phosphorylated full protein. Maris *et al.* (33) used a truncated version of NarL in crystal structure studies because this protein protects exactly the same region and has a longer half-life than the phosphorylated full-length protein.

Proof that the GT nucleotides in the first and second position of the direct repeat are essential was obtained by directed mutation of the first DRU in the promoters of SCO1854 and SCO1993 that were not recognized initially by PhoP^{DBD}. Changes of the GA and CC nucleotides (respectively) to the consensus GT in those positions allowed binding of PhoP^{DBD} leading to the formation

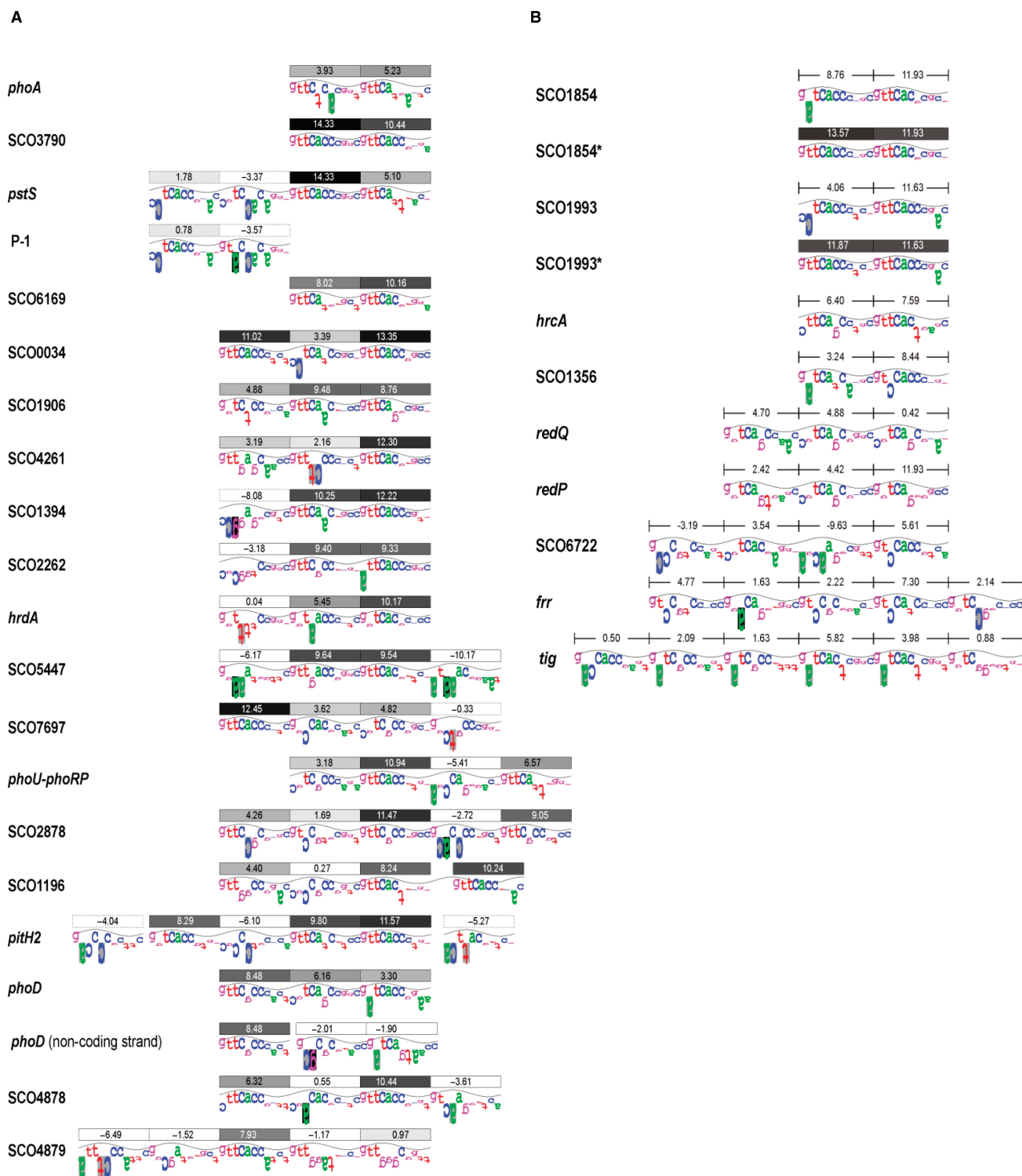


Figure 6. (A) Individual information analysis of the PhoP-binding sites, identified in this and previous works, using the sequence walker method (29). Each binding site is formed with DRUs of 11 nt, which are marked by boxes on its top. Each box contains the individual information (R_i) in bits, and is filled with tones of grey in a scale of 10% steps. Thus, the box of a DRU with a R_i value in the range of 90–100% of the highest possible R_i content (14.63 bits, the R_i of the consensus sequence), are coloured in black; boxes of $R_i \leq 1.46$ bits, are shown in white. Hypothetical DRU, which were not comprised in the footprinting-protected area, are represented by boxes with dashed lines. The height of the letters is the information content in bits, and represents the contribution of each base to the conservation of the sequence. Letters extending downward represent unfavourable contacts. The walker limits are 2 bits, which is also the top of the sine wave, and -3 bits at the bottom; 0 bits is at the middle of the walker. If a base does not occur in the set of sequences that forms the model (model I), a black box is given. A grey box indicates that the upside-down letter extends beyond the lower limit. For simplicity, in this figure the sine wave has a periodicity of 11 bases. (B) Analysis of the promoter-region sequences that resembled PhoP-binding sites, but failed to bind PhoP. The mutagenized SCO1854* and SCO1993* sequences are shown below the wild-type sequences. Information scores (bits) of the sequences are indicated.

of one or two shift bands in the EMSAs. These results confirmed early observations on the importance of the six initial nucleotides, particularly those in positions 1 and 2, in the binding of PhoP^{DBD}. A similar pattern of relevance of the first nucleotides in the PHO boxes has been found in *E. coli*, *B. subtilis*, *Synechocystis*, *Campylobacter jejuni*, or *Corynebacterium glutamicum* (34–38).

A significant number of *S. coelicolor* genes have been found to contain PHO boxes, in agreement with the results of the transcriptomic and proteomic analyses (15). However, there is a considerable variability in the number and organization in the DRus of PHO boxes in those phosphate-regulated genes.

One DRu is not sufficient for clear binding of PhoP^{DBD}. At least two DRus are needed for proper binding of PhoP^{DBD} to a PHO box, since removal of one direct repeat was sufficient to prevent binding of PhoP^{DBD} to a PHO box in the *pstS* promoter. This result is supported by the lack of binding to SCO1854, SCO1993 and *hrcA* promoters, which have a good conserved DRu, but lack a second DRu with a high score.

The basic structure of the 11-nt repeat is important since deletion of 1 nt (the G at position 10 in the 11-nt repeat of the well-known *pstS* promoter) (23) resulted in a drastic reduction of binding of PhoP^{DBD}, as shown by the barely visible formation of PhoP^{DBD}-promoter complex in the shift assays. This result is consistent with the recognition of two direct repeats, at a distance that allows interaction with a PhoP^{DBD} dimer on the same face of the DNA double helix. A similar hypothesis was made for the interaction of the response regulators PhoB and PhoP, from *E. coli* and *B. subtilis*, respectively, with the PHO boxes (6 conserved + 5 variable nucleotides) (32,35). In *E. coli*, the structure of PhoB in complex with its target DNA sequence revealed that PhoB has a winged-helix fold such that helix $\alpha 3$ penetrates in the major DNA groove and a β hairpin wing interacts with the minor groove via the conserved Arg²¹⁹ (Arg²¹⁵ in *S. coelicolor* PhoP). When only two DRus are present, the conservation of the consensus sequence is very important. This property can explain the discrepancy seen with *phoA* (SCO2286), activated by PhoP (28) but with an AA profile in microarray analysis (Table 1, panel A). The time selected for the phosphate shiftdown experiment was chosen on the basis of *pstS* gene expression (15), having a promoter with two highly conserved DRus. *phoA* also has two DRus, but with a lower score, probably requiring a higher concentration of PhoP.

When there are three, four or even more DRus, some of these sequences act as core (C) sequences binding PhoP^{DBD} with high affinity and this allows the subsequent binding of further PhoP^{DBD} molecules, even if those DRus are less conserved.

Three mechanisms explain the sequential binding of PhoP^{DBD} to class III operators. Firstly, the operators of SCO1394, SCO2262, *hrdA*, SCO7697 and SCO5447 contain one or two DRus, named E (extension), with poor sequence conservation adjacent to the core site (Table 1, panel C). Their R_i values are low or negative (Figure 6A). As the information content can be correlated with the binding energy (a positive R_i corresponds to a negative

change of free energy) (27,29), it is proposed that protein-protein interactions stabilize the binding of the PhoP^{DBD} monomer to E repeats. The fast-migrating (lower size) complexes detected in EMSA, should be the result of two PhoP^{DBD} monomers bound to the CC core sites; the slowest complexes should correspond to the full operator-bound complex. This explains the two complexes detected with SCO2262-3 and *hrdA* fragments. Similarly, the three complexes of SCO5447 and SCO7697 fragments (Figure 3; Table 1, panel C) can be explained by a second E direct repeat of still lower conservation (−6.2 versus −10.2 bits in SCO5447; 4.8 versus −0.3 bits in SCO7697; Figure 6A).

A second mechanism can be illustrated with the previously characterized operator of *phoU-phoRP*. It is composed of four DRus: the first two repeats form the core (CC), the third DRu has −5.4 bits of information and was named E_U (for extension unstable) and the last DRu, named E_S (for extension support) is well conserved (Table 1, panel C; Figure 6A). The lack of sufficient conservation of the E_U repeat may prevent the binding of PhoP^{DBD} only to this repeat whereas a binding would take place at both E_U and E_S repeats together. Therefore, the two detected EMSA complexes should correspond to two protein monomers bound to the core (CC) and four monomers bound to the full site, respectively (CCE_UE_S; Figure 3). The same mechanism is proposed for the SCO2878 operator (Table 1, panel C). The third mechanism involves a gap of 1 or 2 nt between the core site and the extension repeat. This gap might act in the same way as an E_U repeat; i.e. deterring the PhoP^{DBD} cooperative binding on DNA. This effect can be quantified by means of the flexible site information equation (39):

$$\text{Site information} = \sum Ri(\text{DRu}) - GS(d) \text{ (bits/site)},$$

where $GS(d)$ is the gap surprisal for the spacing d . The $GS(d)$ term was calculated from the distribution of gap values (44 gaps of 0 nt, two gaps of 1 nt and two gaps of 2 nt; deduced from Table 1), using the gap surprisal equation (39). It results that a gap between two consecutive DRus subtracts 6.4 bits from the site R_i value (both 1- and 2-nt gaps have the same penalty since both have the same frequency). This amount is similar to the R_i of E_U-type repeats (Figure 6A). It follows that the corresponding repeats E_S must add information higher than 6.4 bits to obtain a positive R_i value, as it occurs in SCO1196, *phoU-RP* and SCO2878-binding sites (Figure 6A).

Finally, two putative DRus could have been created in the constructed P-1 promoter when the new restriction sites were introduced. These sequences, located immediately upstream of the DNase-protected site core, would explain the two complexes detected in EMSA (Figures 2 and 4). It is also possible that the wild-type sequences are bound at high regulator concentrations, since they have a sequence similar to the newly introduced one (Figure 6A).

The arrangement of DRus is even more complex in some cases. The operators of *pitH2* and *phoD* are especially complex and appear to use the three mechanisms described above. Furthermore, the *phoD* operator lies in both strands (Table 1, panel C) (28). Analysing the

protected sequences, we observed that in some operators the two DRUs are not strictly adjacent but are separated by 1 or 2 nt. This is the case of SCO1196 and the previously reported *phoD* (28) and *pitH2* (Santos-Beneit, F., unpublished data). In these cases, as in the case of phytase of *B. subtilis* (40), some of the PHO boxes may overlap with the -10 region. At low (or null) PhoP concentration (thus, in 'high' phosphate), this site is poorly or not occupied, whereas at high PhoP concentration (thus, in low phosphate), this site acts as a *cis*-acting negative regulatory site by preventing binding of the RNA polymerase. This seems to happen rarely, since our results and those of *E. coli* and *B. subtilis* clearly indicate that most PhoP-regulated genes are activated by this response regulator (this study; 28).

The *pho* regulon described in this article is of interest as a model for other *Streptomyces* species that are known producers of secondary metabolites with valuable biological activities. Knowledge of the mechanism of phosphate control provides insight into how to increase the production of those secondary metabolites and even to unlock cryptic secondary metabolites (20,41). Moreover, we cannot dismiss that a gene could be regulated by another regulator (probably a repressor) in addition to the activator PhoP. This putative repressor might be abundant and active in Pi sufficiency and absent/inactive in Pi limitation. In consequence, at a given Pi concentration, the expression of a gene would result from the relative abundance/DNA-binding abilities of the positive and negative regulatory factors.

SUPPLEMENTARY DATA

Supplementary data are available at NAR Online.

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