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Short Communication

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PhoB transcriptional activator binds hierarchically to *pho box* promoters

Abstract: The PhoR-PhoB phosphorelay is a bacterial two-component system that activates the transcription of several genes involved in phosphate uptake and assimilation. The response begins with the autophosphorylation of the sensor kinase PhoR, which activates the response regulator PhoB. Upon binding to the *pho box* DNA sequence, PhoB recruits the RNA polymerase and thereby activates the transcription of specific genes. To unveil hitherto unknown molecular mechanisms along the activation pathway, we report biochemical data characterizing the PhoB binding to promoters containing multiple *pho boxes* and describe the crystal structure of two PhoB DNA-binding domains bound in tandem to a 26-mer DNA.

Keywords: PhoB oligomerization state; RNA polymerase sigma 70; transcription activation; two-component signal transduction; X-ray crystal structure.

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Transcription regulation provides bacteria with an effective means to respond to environmental changes. The PhoR-PhoB two-component system, which regulates phosphate uptake and assimilation in *Escherichia coli* and closely-related species, is a well-known example of this kind of response mechanism (Wanner, 1996; Hsieh and Wanner, 2010). Low levels of environmental phosphate (P_i) promote the autophosphorylation of the transmembrane sensor kinase PhoR and, subsequently, the phosphate group is transferred to the response regulator PhoB. The phosphorylation of the PhoB receiver domain ($PhoB^{RD}$; Solà et al., 1999; Arribas-Bosacoma et al., 2007) triggers the transcription of over 50 genes involved in the transmembrane transport of phosphate and the degradation of phosphorous-containing molecules (Pho regulon) (Makino et al., 1989; Kim et al., 2000; Hsieh and Wanner, 2010; Yoshida et al., 2012). The activation is mediated by the PhoB effector domain ($PhoB^{E}$), which binds to a specific DNA sequence formed by two 11-bp direct repeats, the so-called *pho box* (Blanco et al., 2002). Upon binding to DNA, PhoB recruits the RNA polymerase, thereby fulfilling the specific transcription of the Pho regulon genes. The bacterial RNA polymerase consists of a stable core formed by five subunits, two α, β, β′ and ω (Zhang et al., 1999), and is transiently associated with a σ factor, which provides promoter specificity and initiates promoter melting (Wosten, 1998; Murakami et al., 2002; Vassylyev et al., 2002). Among several possible σ factors, σ^{70} is the one responsible for the expression of housekeeping genes in *E. coli*. Canonical σ^{70} promoters contain two conserved hexameric DNA sequences, the -10 and -35 elements; however, in promoters under the control of PhoB, up to three copies of the *pho box* replace the -35 element. σ^{70} is formed by three structural domains (σ_2, σ_3 and σ_4), the C-terminal region (σ_4) being the one that recognizes the -35 element and hence the putative $PhoB^{E}$ target for activating transcription (Makino et al., 1993; Kumar et al., 1994). In a recent study we provided crystallographic evidence that the DNA-bound $PhoB^{E}$ domain directly contacts the σ_4 domain of σ^{70} to accomplish RNA polymerase recruitment (Blanco et al., 2011). Here we complement this finding with biochemical data on the PhoB mode of binding to natural promoters containing multiple *pho boxes* and report a new σ-free crystal structure of the $PhoB^{E}$ dimer bound in tandem to a 26-mer DNA comprising the *pho box* promoter sequence.

To determine the sequence of events following PhoB phosphorylation, we performed a simple size-exclusion chromatography experiment, which confirmed that $PhoB^{E}$ is already a dimer before binding to *pho box* promoters.

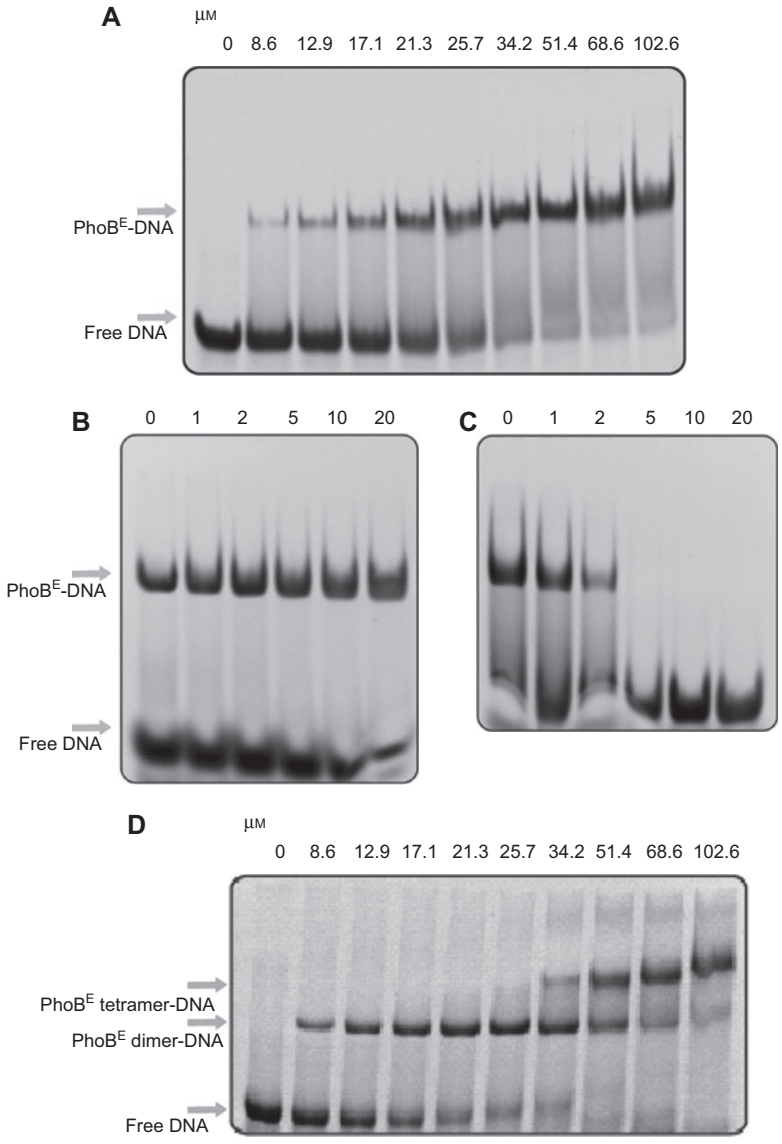

Figure 1 PhoB^E binding to a single *pho box* DNA.
(A) EMSA gel using the labeled DNA fragment encoding the single *pho box* of the *phoA* promoter. Upper numbers indicate the concentration of PhoB^E in μM. (B) EMSA gel with labeled DNA from *pstS pho box* 1. Upper numbers indicate the molar ratio of unlabeled competitor DNA from *pstS pho box* 2. (C) EMSA gel containing the labeled DNA from *pstS pho box* 2. Upper numbers indicate the molar increase of unlabeled competitor DNA from *pstS pho box* 1. (D) EMSA gel using the labeled DNA fragment encoding the double *pho box* of *pstS* promoter. Upper numbers indicate the concentration of PhoB^E in μM. PhoB^E protein expression, purification and DNA complex formation were performed as previously described (Blanco et al., 2002). Each 10 μl-sample of the conventional EMSAs contained 0.1 nmols of previously annealed, fluorescein-labeled dsDNA (at the 5′ end of one of the strands), 0.5 nmols (or 100 nmols, which did not make any difference) of a previously annealed 22-bp competitor dsDNA and variable amounts of PhoB^E. The different components were mixed in binding buffer (20 mM BisTris pH 6.5, 100 mM NaCl, 10 mM $MgCl_2$, 100 μg/ml BSA and 5% glycerol). Samples were incubated for 30 min at 21°C before being loaded onto 8% or 10% polyacrylamide gels (7 cm long, 10 cm wide and 1.5 mm thick) for the 44-bp and 22-bp oligonucleotides (Table 1) respectively, and run in buffer containing 20 mM Tris pH 7.9, 10 mM acetate and 0.1 mM EDTA for 35 min at 4°C. The fluorescence signal was detected by placing the non-denaturing polyacrylamide gels on a conventional UV transilluminator. The band intensity was quantified using the Gene Genome system, with Gene Snap and Gene Tools software (Syngene Bio Imaging, Cambridge, UK). Samples from competitive EMSAs under equilibrium conditions contained 0.1 nmols of labeled dsDNA, 4.3 μg of PhoB^E and increasing amounts of unlabeled competitor DNA from the other *pho box* of the *PstS* promoter. The buffers and protocols remained unchanged.

Under the buffer conditions used, PhoB^E eluted as a single peak corresponding to a relative mass of 25 700 Da, which is equivalent to a PhoB^E dimer (the polypeptide has a globular shape and a molecular weight of 12 583.5 Da, as determined by mass spectrometry). No traces of a second peak corresponding to a monomer were detected, thereby

*phoA*_leading	5′-CTGTCATAAAGTTGTCACGGCC-3′
*phoA*_template[a]	5′-F-GGCCGTGACAACTTTATGACAG-3′
Competitive_leading	5′-TCGGCGACTTTTCGGCGACTTT-3′
Competitive_template	5′-AAAGTCGCCGAAAAGTCGCCGA-3′
*pstS*A_leading	5′-CTGTCATAAAACTGTCATATTC-3′
*pstS*B_leading	5′-ACAGGTGACAGTTATATGTAAG-3′
*pstS*A_template	5′-GAATATGACAGTTTTATGACAG-3′
*pstS*B_template	5′-CTTACATATAACTGTCACCTGT-3′
*pstS*_template[a]	5′-F-CTGTCATAAAACTGTCATAT-TCACAGGTGACAGTTATATGTAAG-3′
*bispstS*B_template[a]	5′-F-ACAGGTGACAGTTATATGTA-AGACAGGTGACAGTTATATGTAAG-3′
*pstS*_leading	5′-CTTACATATAACTGTCACCTG-TGAATATGACAGTTTTATGACAG-3′
*bispstS*B_leading	5′-CTTACATATAACTGTCACCTGT-CTTACATATAACTGTCACCTGT-3′

Table 1 Oligonucleotides used in the EMSA experiments.
[a]*F marks flourescein*.

indicating a protein-protein interaction strong enough to withstand gel filtration.

The *pho boxes* of diverse promoters contain distinct DNA sequences, which result in different PhoB-*pho box* binding affinities. As a first biochemical analysis of $PhoB^E$ binding to a promoter, we tested the binding to an oligonucleotide encoding a single *pho box*. An electrophoretic mobility shift assay (EMSA) was performed using a fixed amount of fluorescein-labeled DNA probe encoding a single *pho box* from the *phoA* promoter with $PhoB^E$ at increasing concentrations. The results systematically showed a single retarded band (Figure 1A). Given that the purified protein eluted as a single peak in a gel filtration column and that no extra bands appeared in the EMSA gels, which would have suggested equilibrium between different stoichiometries and/or oligomerization states, we concluded that the retarded band corresponded to a $PhoB^E$ dimer bound to a DNA molecule.

To analyze the binding of $PhoB^E$ dimers to promoters containing two copies of a *pho box*, we compared the affinity of $PhoB^E$ for the two *pho boxes* of the *pstS* promoter by competition EMSAs under equilibrium conditions, using fluorescein-labeled DNAs encoding either the sequence of the upstream or the downstream *pho box* (*pho box* 1 and 2, respectively). Increasing amounts of unlabeled *pho box* 2 only marginally competed with a labeled *pho box* 1 (Figure 1B). Indeed, a 20-fold excess of unlabeled *pho box* 2 competed only moderately with *pho box* 1 for binding to $PhoB^E$. Accordingly, low amounts of unlabeled *pho* box 1 prevented the binding of labeled *pho box* 2 to $PhoB^E$ (Figure 1C). Thus, during $PhoB^E$ binding to the *pstS* promoter, the protein binds first to *pho box* 1 for which it has a higher affinity. The next step was to check whether binding to *pho box* 1 favors the binding of another $PhoB^E$ dimer to the adjacent downstream *pho box* 2, thereby resulting in a more efficient interaction on a less conserved DNA sequence. An EMSA using the full *pstS* promoter confirmed this. $PhoB^E$ at low concentration readily bound a probe containing the two *pho boxes* of the *pstS* promoter. An increase in the protein concentration produced a slower complex that eventually, at the highest protein concentration, became predominant with neither additional upper bands nor free DNA (Figure 1D). Our interpretation of these two bands, which appeared at a different protein concentrations, is that $PhoB^E$ first binds to the high-affinity *pho box* 1 and afterwards, at higher concentration, binds to the low-affinity *pho box* 2, thus generating the second retardation. A sigmoidal curve (Figure 2A) calculated from the band corresponding to the free DNA running at the bottom of the gel resulted in a dissociation K_{app} of 16 μM, a value lower than that obtained for the *phoA* promoter, which contains only one *pho box*. We speculate that the higher affinity of $PhoB^E$ for the *pstS* promoter is a combined effect caused by the differences between sequences and also cooperativity between the two boxes in *pstS*. To further analyze the influence of *pho box* 1 on binding to *pho box* 2, we performed an EMSA using a DNA fragment with two tandem-repeat *pho boxes* 2 from the *pstS* promoter. Despite the increase in protein concentration, the free DNA band did not disappear (data not shown) and the resulting curve showed slower saturation, indicating a lower affinity of $PhoB^E$ for two consecutive *pho boxes* 2 (Figure 2A) when compared with a combined *pho box* 1-*pho box* 2 tandem. Altogether, our data strongly suggest a cooperative binding mechanism of PhoB to the double *pho box* of the *pstS* promoter (Figure 2B). This type of regulation is reminiscent of the Controller (C) proteins that coordinate the expression of the genes in the type II restriction-modification systems (Tao et al., 1991). Both regulators contain a DNA binding

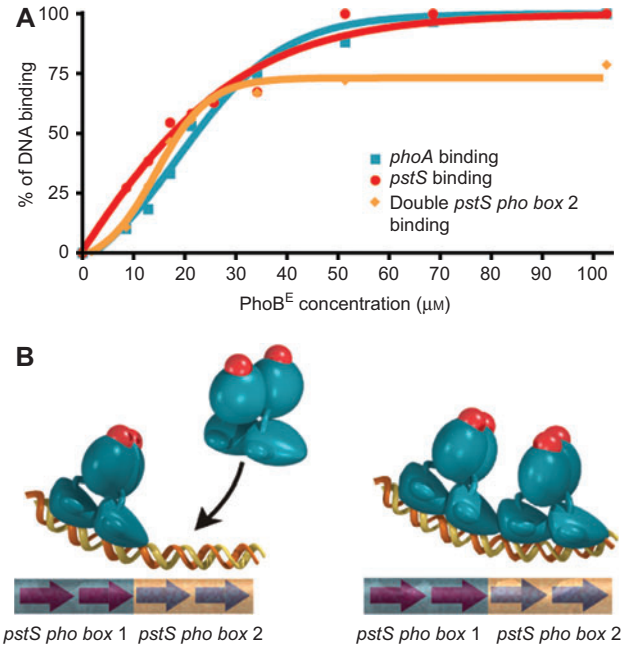

Figure 2 PhoB^E binding to double *pho box* 1, double *pho box* 2 and *phoA* box.
(A) Plot representing DNA binding vs. the concentration of PhoB^E. Resulting sigmoidal curves were fitted through regression analysis. The curve corresponding to the double *pho box* 2 of the *pstS* promoter is not saturated at 100% of DNA binding, but at 70%. The apparent K_{app} is lower for the *pstS* promoter (16 mM) than for the *phoA* promoter (24 mm). (B) Scheme depicting the hierarchical binding of PhoB to the *pstS* promoter, where the PhoB dimer is shown in blue, red spheres denote its phosphoryl groups, and *pho box* 1 and *pho box* 2 containing the two direct repeats are indicated in blue and ivory, respectively.

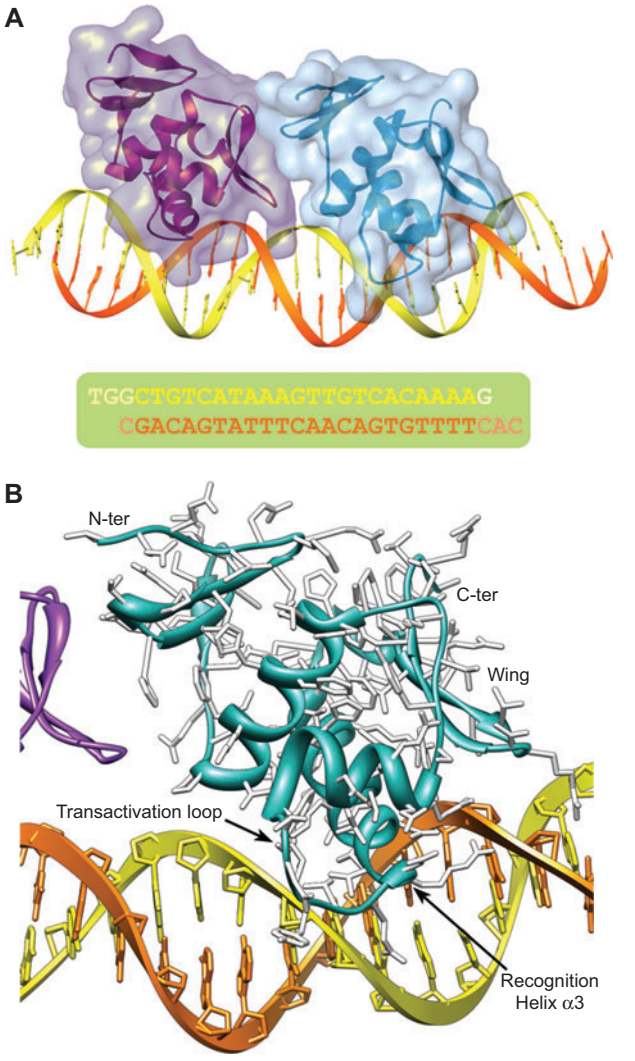

Figure 3 Overview of the PhoB^E-26-mer DNA structure.
(A) Ribbon and surface representation of the PhoB^E-26-mer DNA binary complex structure. The upstream and the downstream protomers of PhoB^E are shown in purple and in greenish blue, respectively. (B) Close-up of the downstream PhoB^E protomer showing the side chains (in white) and the main secondary structure elements involved in DNA recognition. Crystals of the binary complex of PhoB^E and a 26-mer DNA duplex (5′-TGG CTG TCA TAA AGT TGT CAC AAA AG-3′ and 3′-CGA CAG TAT TTC AAC AGT GTT TTC AC-5′) were obtained by mixing a ternary complex solution to 50 mM BisTris (pH 6.5), 100 mM KCl, 10 mM $MgSO_4$ and 17.5% (v/v) PEG 4000. These crystals were cryo-protected in a solution containing 25% glycerol before cryo-cooling in liquid nitrogen. Diffraction data were collected at ESRF beamline ID14-2 to 2.8 Å resolution (Table 2). PhoB^E-DNA crystals contain one PhoB^E dimer in complex with a 26-mer dsDNA molecule per asymmetric unit. The structure was solved by molecular replacement with AMoRe (Navaza, 1994) using the previously solved PhoB^E-DNA model (PDB code 1GXP; Blanco et al., 2002). Manual model building followed with Turbo (Carranza et al., 1999), using σ_A-weighted Fourier maps, with coefficients $2F_{obs}-F_{calc}$ and $F_{obs}-F_{calc}$, alternated with automatic positional and temperature factor refinement with CNS (Brünger et al., 1998). The data used in the refinement process were corrected for bulk-solvent and anisotropy (Table 2).

helix-turn-helix motif and bind as dimers to promoters with two dimer-binding sites with different affinity. A hierarchy of binding strength to individual sites, together with cooperativity between the binding dimers, ensures a sharp response of the regulated promoter (Bogdanova et al., 2009).

In addition to these biochemical data, we confirm the mode of PhoB binding to DNA with a new crystal structure of PhoB^E in complex with a 26-mer *phoA pho box* encoding DNA, which was solved and refined to 2.8 Å resolution. The PhoB^E dimer binds to the 26-mer double-stranded oligonucleotide in tandem, an arrangement very similar to that previously reported by our group using a different 23-bp oligonucleotide (Blanco et al., 2002). This observation thus confirms the protein and DNA interactions within the complex (Figure 3A). A PhoB^E protomer consists of an N-terminal four-stranded antiparallel β-sheet (strands β1–β4), followed by a compact three α-helix bundle (helices α1–α3) packed against a small C-terminal

Data collection	
Wavelength (Å)	0.933
Space group	$C222_1$
Cell parameters (Å)	a=124.6
	b=165.8
	c=59.9
Resolution range (Å)[a]	81.7–8.0/3.0–2.8
Number of measurements	128 173
Number of unique reflections	14 275
R_{merge}[a,b]	6.2/23.2
Completeness[a]	91.1/53.3
$\langle I \rangle / \langle \sigma(I) \rangle$[a]	8.1/3.1
Multiplicity[a]	4.0/2.1
Refinement	
Resolution range (Å)	50.0.-2.8
Number of reflections used	13537
Number of protein and DNA atoms	2749
Number Ca^{2+} atoms	2
rmsd for bonded angles (°)	2.002
rmsd for bond lengths (Å)	0.015
rmsd for bonded B-factors (Å²)	0.385/1.016
(main-chain/side-chain)	
R_{factor}/R_{free} (%)[c]	24.6/28.5
Mean B-value (Å²) (protein/DNA atoms)	63.2/78.0

Table 2 Data collection and refinement statistics of the $PhoB^E$/26-mer DNA structure.

[a]Overall/outermost resolution shell.

[b]$R_{merge} = [\Sigma_{hkl} \Sigma_i |I_i(hkl) - \langle I_i(hkl) \rangle| / \Sigma_{hkl} \Sigma_i I_i(hkl)] \times 100$ where I_i is the ith measurement of reflection hkl.

[c]$R_{factor} = [\Sigma_{hkl} ||F_{obs}| - k|F_{calc}|| / \Sigma_{hkl} |F_{obs}|] \times 100$; R_{free} same for 5% of reflections not used during refinement.

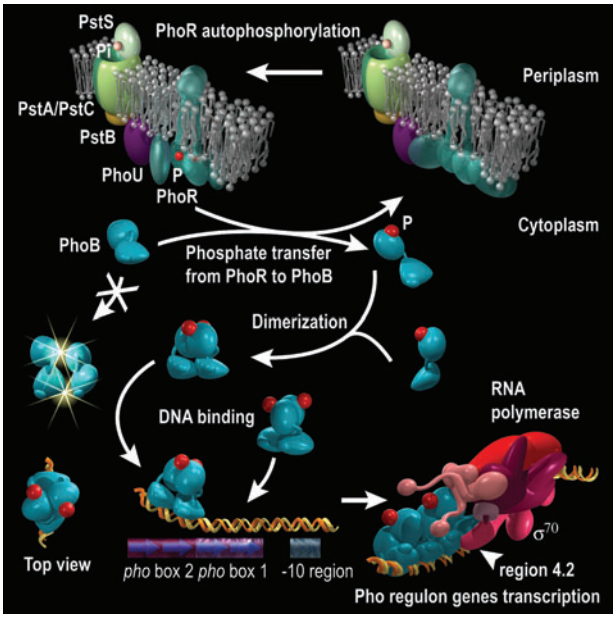

Figure 4 Proposed model for $PhoB^E$ binding to DNA and RNAPH recruitment.

Summary diagram based on our experimental results, showing the PhoB dimerization states after phosphorylation by PhoR, the hierarchical binding to the DNA *pho boxes* and the transcriptional activation based on RNAPH recruitment by direct contact with the σ_4 domain of the σ^{70} subunit (see text for further details).

β-sheet, which includes a β-hairpin or 'wing' (Figure 3B). $PhoB^E$ has a winged-helix motif for DNA binding, where helix α3 is the recognition helix. Between helices α2 and α3 within the modified helix-turn-helix motif, there is a seven-residue loop which has been named the transactivation loop because it was postulated to interact with the σ^{70} subunit to activate transcription (Makino et al., 1996; Blanco et al., 2002); we recently confirmed this theory with the crystallographic structure of a ternary complex formed by the $PhoB^E$ dimer, a *pho box* DNA and the σ_4 domain of σ^{70} (Blanco et al., 2011). In the present structure, a tandem of two protomers arranged head-to-tail sits at one side of the DNA molecule, the recognition helices α3 lay along the major groove while the wing tips contact the downstream minor groove, performing specific (with bases) and non-specific (with phosphates) interactions (Figure 3B). In this 26-mer structure, the DNA spacing, which is longer than in the former 23-mer structure, does not allow a pseudo-continuous protein-DNA fiber, where each bound tandem contacted the neighboring proteins. The DNA is especially bent at the edges, about 29°, which is slightly less than the previous complex. The N-terminal β-sheet and the wing of the upstream and downstream protomers present slight changes with respect to the 23-mer complex structure, such as different conformations in the side-chains of residues, which contacted neighboring $PhoB^E$ molecules in the continuous tandem arrangement in the 23-mer complex.

In accordance with the EMSA assays, our structural data indicate that the cooperative binding is directed by protein-protein interactions between adjacent $PhoB^E$ dimers. The comparison of the $PhoB^E$-DNA arrangement in the complex with a 23-bp DNA-mer (Blanco et al., 2002), which forms pseudo-continuous protein-DNA fibers, with the one in complex with a 26-bp DNA-mer (this work) reveals that the higher DNA bending in the former promotes contacts between adjacent $PhoB^E$ dimers. A previous *in vivo* study on transcription using *pho box* deletions of the *ugpB* promoter fused to a reporter gene also gave clues about hierarchical and cooperative binding (Kasahara et al., 1991). Although the authors did not relate their findings to cooperative binding, their results showed that at cellular concentrations of PhoB the intermediate *pho box* was required for the protein to bind to the downstream box and activate transcription.

The combination of our biochemical and structural data with previously reported information allows a finer description of the whole path leading to the RNA

polymerase recruitment by PhoB bound to the promoter (Figure 4). Low levels of environmental phosphate stimulate the autophosphorylation of the membrane-attached sensor kinase PhoR. The phosphate group is then transferred to the receiver – or regulatory – domain of PhoB, which is thereby activated. Activation promotes PhoB dimerization, a process that must take place before binding to DNA, as in our experiments the PhoB effector domain $PhoB^E$ binds as a previously formed dimer to the *pho box per se*, with no need of the regulatory domain. The EMSA assays demonstrate that two $PhoB^E$ dimers bind to two consecutive *pho boxes* in a hierarchical and cooperative manner. In the *pstS* promoter, which contains two *pho boxes*, a first PhoB dimer binds the upstream *pho box*, which has higher sequence conservation and affinity and acts as a primer for the subsequent dimer to bind to the neighboring box positioned downstream. The lower affinity of PhoB for this second sequence is compensated by protein-protein interactions between dimers, more likely when the DNA is bent (Blanco et al., 2002) than when it is in a more extended form (as in the current structure). A hierarchical binding was already described for the ortholog OmpR and its functional relevance in the alternative activation and repression of the genes that code for two porins to control cellular osmolarity was deciphered (Slauch and Silhavy, 1989; Rampersaud et al., 1994; Huang et al., 1997; Head et al., 1998; Yoshida et al., 2006). PhoB binding to the *pho box* promoter sequence leads to the recruitment of RNA polymerase. The DNA-PhoB complex improves the interaction with the σ_4 domain of σ^{70} (Blanco et al., 2011) and hence PhoB mediation effectively triggers the expression of the specific genes belonging to the Pho regulon and phosphate uptake is fulfilled.

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