Fine-Tuning Control of phoBR Expression in Vibrio cholerae by Binding of PhoB to Multiple Pho Boxes

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The control of Vibrio cholerae phoBR expression by PhoB involves its binding to Pho boxes at −35 (box 1), −60 (box 2), and −80 (box 3) from the putative phoBR translation start site. These loci were located in the sense (box 1) and antisense (boxes 2 and 3) strands of the phoBR regulatory region, and PhoB binds to these individual boxes with distinct affinities. Fusions of sequences containing different combinations of these boxes upstream of the lacZ reporter in a plasmid demonstrated that only those carrying boxes 1, 2, and 3, or 1 alone, activated transcription under inorganic phosphate (Pᵢ) limitation. When a fragment, including only boxes 1 and 2, was fused to lacZ, expression was no longer induced by low Pᵢ, suggesting a repressive role for PhoB−box2 (PhoB bound to box 2) over the transcriptional activity induced by PhoB−box1. The similarity between lacZ expression levels from promoter fragments containing the three boxes or box 1 alone showed that PhoB−box3 eliminated the repressive effect imposed by PhoB−box2 on phoBR transcription. Complementation assays with a phoBR-containing plasmid demonstrated that the 234-bp promoter fragment carrying the three boxes is absolutely required for operon expression in Vibrio cholerae ΔphoBR cells. This was observed under Pᵢ abundance, when phoBR was expressed at a basal level and, also in low Pᵢ conditions, when Pho regulon genes were fully expressed. Thus, under Pᵢ limitation, PhoB exerts dual regulatory functions by binding sequentially distinct Pho boxes to enable the fine-tuning and precise control of phoBR expression in V. cholerae cells.

Vibrio cholerae is found most commonly in marine or estuarine habitats, free or in association with phytoplankton and zooplankton and other marine organisms (7). Certain strains, mainly of serotypes O1 and O139, can be pathogenic to humans and cause cholera, an acute diarrheal disease that is responsible for significant mortality and economical damage in several parts of the world (6).

To establish a successful infection, V. cholerae must survive the stomach acidic environment, reach the small intestine, and synthesize TCP, the colonization factor toxin-coregulated pilus, and then CT, the cholera toxin (4, 11, 19, 20, 32, 44), among other factors. The expression of TCP and CT is transcriptionally regulated by ToxT, a member of the ToxR-dependent cascade, in response to environmental stimuli (34).

To survive and multiply in environments as diverse as aquatic ecosystems and the host digestive tract, V. cholerae coordinately regulates gene expression to generate a suitable adaptive response. Aquatic and terrestrial ecosystems generally are poor in nutrients such as phosphorus (46), an essential element since it is a constituent of phospholipids, phosphoproteins, and nucleic acids, and it is involved in most metabolic pathways as ATP, inorganic phosphate (Pᵢ), or coenzymes.

In many bacterial species, phosphorus-containing compound uptake and metabolism depend on the extracellular Pᵢ concentration. Under Pᵢ starvation conditions, these processes require a two-component regulatory system to couple stimulus-response mechanisms, favoring bacterial cell adaptation to the environment (12). In Escherichia coli, V. cholerae, and other species, the system is encoded by the phoBR operon, and in Bacillus subtilis and Streptomyces coelicolor it is encoded by its homologues phoPR and phoRP, respectively (18). PhoR is the sensor histidine kinase that monitors extracellular Pᵢ availability, and PhoB (or PhoP) is the response regulator, which transcriptionally regulates a set of genes, the Pho regulon, that is involved in bacterial phosphate management (12, 13). Recent studies have shown that the PhoBR system has many additional roles in bacteria (12, 13, 18). In particular, roles for PhoBR have been demonstrated in V. cholerae motility, biofilm formation, intestinal colonization, and virulence (9, 10, 36, 37, 43, 47).

E. coli response to phosphate deficiency involves PhoR autophosphorylation and the transfer of Pᵢ to its cognate response regulator, PhoB (26). Phosphorylated PhoB (PhoB−Pᵢ) binds with higher affinity than the nonphosphorylated protein to conserved 18-bp DNA sequences, called Pho boxes, in the regulatory regions of Pho regulon genes (28). The E. coli consensus Pho box, CT(GT)CAT A(AT)A(AT) CTGTC(A), consists of two 7-bp direct repeats separated by a conserved

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4-bp AT rich spacer, where PhoB binds as a head-to-tail dimer and interacts with the RNA polymerase σ70 subunit to control the transcription of downstream genes (3, 25). Most Pho regulon promoters contain a single Pho box that is sufficient to confer regulation. However, PhoB-binding sites can be found in two or more copies within some promoter regions (15–17), where the protein usually binds in a cooperative manner for the transcription of downstream genes (3, 25). Most Pho regulon genes constitute an operon whose regulatory region is autoregulated by a novel and complex mechanism underlying its expression. In this work, we demonstrated that the V. cholerae phoBR operon is autoregulated by a novel and complex mechanism that involves PhoB binding to three Pho boxes, at −35 (box 1), −60 (box 2), and −80 (box 3) upstream of the putative phoB translation start site. These boxes are located in the sense (box 1) and antisense (boxes 2 and 3) strands, and PhoB interacts with them in a sequential and cooperative manner and with different affinities. PhoB bound to distinct boxes acts as a transcriptional activator or a repressor, fine-tuning the expression of the phoBR operon under P_i limitation. Furthermore, we provide evidence that phoBR is transcriptionally activated by proteins other than PhoB when cells are cultivated under P_i limitation.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and culture conditions.** Bacterial strains and plasmids used in this work are listed in Table 1. Cells were routinely cultured in Luria-Bertani (LB) medium supplemented with 100 μg/mL ampicillin (Ap), 50 μg/mL kanamycin (Km), or 30 μg/mL chloramphenicol (Cm) as appropriate. Plasmids pGEX4T-3 and pIC86mut were used as high-copy vectors containing a putative transcription start site regulating lacZ with 86-bp upstream of phoBR. All other plasmids used in this work are listed in Table 1.

**Plasmids**
- pGEX4T-3: High-copy vector containing an MCS preceded by GST coding region; Ap
- pIC552: lacZ preceded by an MCS to insertion of a promoter region; Ap
- pIC525 containing a region of 234 bp upstream of phoBR putative transcription start site regulating lacZ; Ap
- pIC86: pIC525 containing a region of 86 bp upstream of phoBR putative transcription start site regulating lacZ; Ap
- pIC262: pIC525 containing a region of 62 bp upstream of phoBR putative transcription start site regulating lacZ; Ap
- pIC264mut: pIC525 containing the mutated region of 86 bp upstream of phoBR putative transcription start site regulating lacZ; Ap
- pIC86mut: pIC525 containing the mutated region of 86 bp upstream of phoBR putative transcription start site regulating lacZ; Ap
- pWKS30: Low-copy vector; Ap
- pGEM-T Easy: High-copy easy cloning vector; Ap
- pWK5: High-copy plasmid; contains phoBR operon, including entire regulatory region; Ap
- pWK20: 3 kbp pWK5 PstI fragment inserted into PstI pWKS30 restriction site; contains phoBR coding region plus 234-bp promoter; Ap
- pCG7: pWK5 containing a SacI restriction site in the center of phoBR regulatory region; Ap
- pCG8: 3-kbp pCG7 PstI fragment inserted into PstI pWKS30 restriction site; contains phoBR coding region plus 234-bp mutated promoter; Ap
- pLC1: pWK5 reverse amplified, digested with BglII, and linked; contains phoBR plus 62-bp promoter; Ap
- pLC2: pWK5 reverse amplified, digested with BglII, and linked; contains phoBR plus 86-bp promoter; Ap
- pLC3: 2.3-kbp pLC1 BglII/PstI fragment, inserted into BamHI/PstI pWKS30 restriction sites; contains phoBR plus 62-bp promoter; Ap
- pLC4: 2.3-kbp pLC2 BglII/PstI fragment, inserted into BamHI/PstI pWKS30 restriction sites; contains phoBR plus 86-bp promoter; Ap
- pLC5: 2.3-kbp fragment of pCG7 amplified and cloned into vector pGEM-T Easy; contains phoBR plus 86-bp mutated promoter; Ap
- pLC6: 2.3-kbp pLC5 NotI fragment, inserted into NotI pWKS30 restriction site; contains phoBR plus 86-bp mutated promoter; Ap

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<td>Classical Inaba, Sm', wild-type V. cholerae</td>
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<tr>
<td>WK3</td>
<td>ΔphoBR mutant of 569BSR; Sm' Km'</td>
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<td><strong>Plasmids</strong></td>
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<td>pIC552 containing a region of 86 bp upstream of phoBR putative transcription start site regulating lacZ; Ap</td>
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<td>pLC6</td>
<td>2.3-kbp pLC5 NotI fragment, inserted into NotI pWKS30 restriction site; contains phoBR plus 86-bp mutated promoter; Ap</td>
<td>This work</td>
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*a Sm, streptomycin; Km, kanamycin; Ap, ampicillin; Cm, chloramphenicol; MCS, multiple-cloning site; ORF, open reading frame.*
propagated in LB agar or LB for liquid cultures (39). The minimal medium TG (V. cholerae, E. coli) was used as a template for subsequent amplification with the primers and specific primer sets. For double-stranded probes, six oligonucleotides were used to generate three pairs: PhoBNcoI (5'-GGCGAAGATCTGCTGTAACGCGCCGCTCGAGTTAG-3'), PhoBXhoI as well as P170Fmut (5'-GGCGGATCCGAGAAATGCTTACTT-3') and CPMut2 (5'-ATTACCCGGTGTCACGGCGCCCGGAAATTCC-3'). The 2.3-kb fragment containing the regulatory region upstream of the lacZ gene into pIC552 that was digested with NcoI and XhoI, cloned into pGEX4T-3 (GE Healthcare) digested with the same enzymes, generating pGEXphoWT. The purity and concentration of the recombinant PhoB was determined by densitometric analysis of a Coomassie-stained SDS-PAGE 12.5% gel using the software Scion Image (IBM PC) and by measuring the OD280 (39). The soluble 26-kDa purified PhoB was trypsin digested (40) and analyzed by mass spectrometry on a Voyager DE PRO Biospectrometry workstation (Applied Biosystems). Samples containing purified PhoB were aliquoted and stored at −70°C.

Search for putative Pho box sequences upstream of phoB. For a search for Pho box-like sequences within the 234-bp phoB regulatory region was performed in both genomic and antisense orientation using the GLAM2CAN tool (8), followed the MEME suite (2), based on the consensus E. coli Pho box motif (24), which was constructed with the GLAM2 tool.

EMSAs. Electrophoretic mobility shift assay (EMSA) probes were duplex DNA oligonucleotides prepared either by annealing complementary single-stranded DNA oligonucleotides or by PCR using pWK5 or pCG7 (Table 1) as templates and specific primer pairs. For double-stranded probes, six oligonucleotides were used to generate three pairs: cx1fwd (5'-GGCGATGCTGTAACGCGCCGCTCGAGTTAG-3') and cx2rev (5'-GGATCCGGTTCACGGCGCCCGGAAATTCC-3') and cx3fwd (5'-GGCGGATCCGAGAAATGCTTACTT-3') and cx3rev (5'-ATTACCCGGTGTCACGGCGCCCGGAAATTCC-3'). Each primer pair was incubated in annealing buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA) (39) under the following conditions: 88°C for 2 min, 65°C for 10 min, 37°C for 30 min, and 25°C for 5 min. The PCR products and the annealed double-stranded oligonucleotides were purified from unincorporated nucleotides (39), quantified, and radioabeled at the 5' end with [-32P]ATP using T4 poly-nucleotide kinase (Invitrogen) (39). The binding of the purified PhoB to DNA probes and analysis of the products by gel electrophoresis were carried out as described previously (31). Reaction mixtures of PhoB with the 234-bp phoB regulatory region contained 50 fmol of probe and increasing concentrations of PhoB (from 0 to 32 μM) in binding buffer (20 mM Tris-HCl, pH 7.0, 50 mM NaCl; 1 mM dithiothreitol [DTT]; 10 mM MgCl2; 100 μM bovine serum albumin [BSA]; 0.5 μg/ml salmon sperm DNA) in a total volume of 10 μl. Competition assays were performed using 50 fmol of radiolabeled promoter fragment, PhoB at 32 μM, and 50, 250, or 500 fmol of unlabeled probe. Comparative EMSAs with 234-, 172-, and 62-bp fragments were performed using 50 fmol of labeled probes and increasing PhoB concentrations (0.5, 2.5, 5, 10, 20, 30, and 37 μM). The specific ligation of PhoB to these individual sequences was tested using an excess of unlabeled probes (data not shown). EMSAs with sequences containing individual Pho box 1, 2, or 3 were carried out with 50 fmol of each probe and increasing concentrations of PhoB (from 0 to 37 μM). For competition assays, 50, 250, and 500 fmol of nonlabeled oligonucleotides was added to reaction mixtures containing 30 μM PhoB and 50 fmol of the corresponding labeled probe. To assess the relative binding affinity of PhoB for the individual Pho box 1, 2, or 3, reactions were carried out with 50 fmol of labeled Pho box 1 and PhoB at concentrations ranging from 0 to 30 μM (control). Another set of reaction mixtures contained PhoB at 30 μM, 50 fmol of labeled Pho box 1, and a total of 500 fmol of nonlabeled competitor probes as equimolar mixtures of Pho boxes 1, 2, and 3; Pho box 1; Pho box 2; and Pho box 3. All reaction mixtures were incubated at room temperature for 30 min and then subjected to electrophoresis on a 7% nondenaturing polyacrylamide gel in 1×
Tris-acetate-EDTA buffer (39). Gels were exposed to a Phosphomager screen (Molecular Devices) casee sealed against light for 24 h.

**DNase I footprinting.** The oligonucleotide PhoBXhol was radiolabeled at the 5′ end using [γ-32P]ATP and T4 polynucleotide kinase (39). After labeling, the oligonucleotide was precipitated and resuspended in diethyl pyrocarbonate (DEPC)-treated water. The 32P-labeled oligonucleotide PhoBXhol and nonlabeled PhoBNCol oligonucleotide were used as primers to amplify the 234-bp sequence upstream of phoBR using pWKS (wild-type promoter) and pCG7 (mutant promoter) as templates. The 234-bp fragments were purified and quantified as described before. G+A sequencing (obtained by the chemical digestion of DNA) was carried out with 234-bp wild-type and mutated fragments. The reaction products were resuspended in 5 µl of sequencing buffer (17 sequencing kit; GE Healthcare) and subjected to gel electrophoresis alongside DNase I digestions products (39).

Purified *V. cholerae* PhoB (0.5, 2.5, and 10 µM) was incubated with 200 fmol of wild-type or mutated 234-bp DNA probe in binding buffer in a total volume of 49 µl. Reaction mixtures were incubated for 30 min at room temperature, and then 0.01 µl of DNase I was added per tube for probe digestion. Reaction tubes were left at room temperature for 1 min, after which 140 µl of stop solution (192 mM sodium acetate, 32 mM EDTA, 0.14% SDS, 64 mg/ml herring sperm DNA) were added in each tube. DNA was extracted with phenol-chloroform and precipitated with sodium acetate and ethanol (39). The DNA pellet was resuspended in 5 µl of sequencing buffer. Products of DNase I digestion, as well as G+A sequencing, were subjected to electrophoresis alongside DNase I digestions products (39).

**Enzymatic assays.** For promoter activity assay, *V. cholerae* wild-type (569BSR) and ΔphoBR mutant (WK3) strains containing plasmid pIC552, pIC234, pIC86, pIC62, pIC234mut, or pIC68mut were grown in LB medium until an OD 600 of 0.5. Cells were collected by centrifugation, resuspended in the same volume of TGH (P-abundance) or TGLP (P-limitation), and cultivated for 16 h, at which time the promoter transcriptional activity was assessed by measuring β-galactosidase activity (9). PhoA (alkaline phosphatase) activity, the control of Pho regulation, was determined as previously described (47) in the same cells. Activities of both enzymes were expressed in U/µg of protein (22).

For complementation assays, *V. cholerae* ΔphoBR mutant (WK3) cells containing plasmid pWK20, pCG8, pLC3, pLC4, or pLC6 were cultivated as described above, and PhoA activity was determined as previously described (47).

**RESULTS AND DISCUSSION**

**PhoB binds to multiple sites in the *V. cholerae* phoBR regulatory region in vitro.** In *E. coli*, the expression of the phoBR operon is autoregulated by PhoB binding to a single Pho box at −35 of the transcription initiation site of phoBR, as previously described (45).

To investigate whether a similar regulatory mechanism occurs in *V. cholerae* cells, we performed EMSA using purified *V. cholerae* PhoB and a 32P-radiolabeled PCR fragment of 234 bp corresponding to the sequence immediately upstream of phoBR. The experiments were carried out with the unphosphorylated form of the protein, since it has been shown that *E. coli* PhoB does bind to the same Pho boxes in vitro but with lower affinity than its phosphorylated form (31). PhoB retarded the fragment mobility even at 1.5 µM, giving rise to two shifted bands in the EMSA gel (Fig. 1b). When PhoB was used at higher concentrations, a third complex was formed, which was represented in the gel by a very slow mobility band whose intensity increased with the protein concentration. EMSA with an unlabeled 234-bp fragment as the competitor DNA fragment showed the displacement of the labeled probe bound to PhoB, confirming the specificity of the protein-DNA interaction (Fig. 1b, right). The binding of purified PhoB to the phoBR regulatory region explains why in earlier EMSAs using protein extracts, only those from wild-type *V. cholerae* cells grown under P− limitation shifted the phoBR 234-bp promoter fragment (data not shown).

Differently from *E. coli*, where the expression of phoBR is controlled by PhoB binding to a single Pho box (45), these results suggest that the *V. cholerae* phoBR regulatory region has multiple sites with different binding affinities for PhoB. In line with our results, it has been demonstrated that the expression of homologue operons, such as phoRP of *Streptomyces coelicolor* (41) and phoPR of *B. subtilis* (33), also requires the binding of the response regulator PhoP to multiple Pho boxes in their regulatory regions for efficient transcription.

**Three putative Pho boxes were identified in the phoBR regulatory region.** A similarity search based on the *E. coli* Pho box consensus sequence (28) was carried out with the 234-bp regulatory region of *V. cholerae* phoBR using GLAM2 and GLAM2SCAN tools (2, 8). Three candidate sequences were found, at approximately −35 in the sense orientation and at −60 and −80 in the antisense orientation. Their sequences, positions, and orientations relative to the putative phoB translation initiation site are shown in Fig. 2a and b.

To confirm the location of PhoB binding sites in the phoBR regulatory region, a DNase I protection footprinting assay was performed (Fig. 2c). PhoB protected the DNA segment spanning nucleotides 20 to 90 from DNase I, which included three adjacent putative binding sites at −35, −60, and −80, with different binding affinities to PhoB (Fig. 2c, left). These sites correspond exactly to those identified by the PhoB binding motif search (Fig. 2a and b). It is interesting that relative binding affinities of PhoB to these sites seem to be in agree-
ment with the identity level of each one to the E. coli consensus Pho box (Fig. 2b). The PhoB binding site at −35 corresponds to the sequence predicted in a previous work (47), with extensive identity (−80%) to the Pho box in the regulatory region of the E. coli phoBR operon (28). These putative PhoB binding sites are referred to as boxes 1 (−35), 2 (−60), and 3 (−80).

Binding of PhoB to phoBR Pho boxes in vitro seems to be sequential and cooperative. Since the three Pho boxes are in close proximity to each other in the phoBR regulatory region, the hypothesis of sequential and cooperative binding of PhoB to these sites was tested. To this end, a footprinting assay was performed as described earlier, using as a probe the 234-bp mutated fragment within the AT-rich core of Pho box 2. More precisely, the sequence TTAT was replaced by GCGG within Pho box 2 (Fig. 2b), because this AT-rich spacer between the two 7-bp direct repeats has a functional role in the intrinsic bending of the Pho box sequence upon PhoB binding in E. coli (24, 25). Thus, we expected to disturb the interaction of PhoB with box 2 and analyze its overall effect on PhoB binding to the phoBR regulatory region.

Interestingly, this mutation did not affect PhoB binding to box 1 but prevented the binding of the protein to boxes 2 and 3, even at higher protein concentrations (Fig. 2c, right). These findings suggested that the binding of PhoB to box 3 in vitro is dependent on the prior binding of the protein to box 2.

It has been shown that the expression of some E. coli Pho regulon genes, namely, psiE (16), ugpB (15), and psiS (17, 27), depends on the cooperative binding of PhoB to tandemly arranged Pho boxes in their regulatory regions. Such an arrangement seems to be required for the effective binding of PhoB to weakly conserved Pho box sequences, such as those in psiE and ugpB promoters, to enhance transcriptional activation (16). On the other hand, there are Pho regulon genes whose expression is repressed by the binding of PhoB to Pho boxes in their regulatory regions. This has been described, for instance, for the operons tagAB and tagDEF of B. subtilis, whose expression is both positively and negatively controlled by the two-component signal transduction system PhoPR, a homologue of E. coli PhoBR (13). In such cases, PhoP binding to Pho boxes is required for the transcriptional activation and/or repression of the operons under P$_i$ starvation. Interestingly, some of these PhoP binding sites are located in the antisense strand of the promoters (21). Therefore, under P$_i$ starvation, the activity of the Pho regulon in both E. coli and B. subtilis is modulated by the binding of their corresponding response regulators to Pho boxes to elicit specific responses to P$_i$ limitation, which might involve the induction or repression of gene expression.

The putative PhoB binding sites identified in the V. cholerae phoBR regulatory region are located in the sense (box 1 at −35) and antisense strands (box 2 at −60 and box 3 at −80).
Therefore, the binding of PhoB to these sequences under Pi limiting conditions might result in the activation and/or repression of phoBR to fine-tune the transcription of Pho regulon genes. To further explore the interaction between *V. cholerae* PhoB and Pho boxes, we performed EMSAs with purified protein and three distinct double-stranded oligonucleotide probes, corresponding to the sequences of the individual Pho boxes (Fig. 3). At the lowest concentration tested (2 μM), PhoB retarded the electrophoretic mobility of the box 1 (−35)-containing probe only and did not bind to Pho box 2 or box 3 probe, even at the highest concentration used (37 μM) (Fig. 3a, b, and c).

The relative binding affinity of PhoB to the three Pho boxes was further analyzed by competition EMSA. To this end, the labeled complex of PhoB fused to box 1 (PhoB−box1) was incubated with unlabeled oligonucleotides corresponding to box 1 itself or box 2 or 3 to test their ability to displace the bound protein from box 1. Only unlabeled Pho box 1 containing probe and the mixture of the three phoBR Pho boxes effectively displaced PhoB from the complex (Fig. 3d). These results confirmed the higher binding affinity of PhoB to box 1 relative to boxes 2 and 3. They also showed that box 1, at −35, was the only one within the phoBR regulatory region that functioned independently as a PhoB binding site (15).

Additional EMSAs (Fig. 4a, b, and c) were performed to compare the binding of PhoB to phoBR promoter regions containing distinct combinations of the three Pho boxes. The PhoB binding patterns to fragments with boxes 1, 2, and 3 (Fig. 4a) and to Pho box 1 only (Fig. 4c) confirm the results presented before (Fig. 1b and 3a, respectively).

Although PhoB did not bind in vitro to probes containing Pho box 2 or 3 separately (Fig. 3b and c, respectively), it did bind to the promoter version containing boxes 2 and 3 (Fig. 4b). However, the mobility shift of this fragment was observed only at PhoB concentrations above 5 μM, about 10-fold higher than that required to retard fragments with boxes 1, 2, and 3 or box 1 (Fig. 4a and c), thus confirming the hypothesis that the binding of PhoB to boxes 2 and 3, in this order, is somehow assisted by the complex PhoB−box1.

Taken together, these results suggest that (i) PhoB presents higher binding affinity in vitro to box 1 than to box 2 or 3, and (ii) the binding of PhoB to Pho boxes 1, 2, and 3 in vitro occurs in a sequential order and seems to be cooperative, as described for the PhoB-dependent regulation of the *E. coli* gene *ugpB* (15).

**Binding of PhoB to Pho boxes in the sense and antisense strands of phoBR promoter region fine-tunes the expression of the operon.** To test whether the in vitro binding pattern of PhoB to phoBR Pho boxes matches the in vivo binding of the protein to the phoBR regulatory region and correlates with promoter activity, wild-type 569BSR and its isogenic phoB mutant, WK3, were transformed with plasmids containing the reporter gene *lacZ* under the control of distinct combinations of Pho boxes 1, 2, and 3 (Fig. 1a). Promoter activities were estimated by β-galactosidase levels in transformed cells grown under Pi limitation (TGHP medium) or Pi abundance (TGLP medium) for 16 h, when the expression of both the phoBR
operon and Pho regulon (measured by alkaline phosphatase expression) still represents peak levels (data not shown).

Similar β-galactosidase activity levels were observed in wild-type cells, grown under P_7, limitation (LP), harboring either boxes 1, 2, and 3 fused to lacZ (box1,2,3−lacZ) or box1−lacZ (Fig. 5a). Under P_7, abundance (HP), wild-type cells with box1,2,3−lacZ showed low levels of β-galactosidase activity, while wild-type cells carrying box1−lacZ responded similarly to those harboring promoterless lacZ (Fig. 5a). These data suggest that phoBR expression from Pbo box 1 is strictly dependent on P_7, limitation in vivo. However, the full-length phoBR regulatory region (with boxes 1, 2, and 3; 234 bp) apparently responds to other stimuli, which could explain its small but detectable in vivo activity in P_7, rich medium, in agreement with previous findings (9, 10, 36, 47, 48).

Surprisingly, wild-type cells carrying box1,2−lacZ cultured either in P_7, limitation (LP) or P_7, abundance (HP) showed virtually no β-galactosidase activity, indicating the repression of lacZ transcription. In comparison, wild-type cells harboring box1,2mut−lacZ presented low levels of β-galactosidase activity, but only under P_7, limitation (LP), suggesting that transcription from the fragment with boxes 1 and 2 is a PhoB-dependent process and that the mutation in box 2 positively affected the transcription of phoBR (Fig. 5a).

The low level of β-galactosidase activity in wild-type cells carrying the construct box 1 and mutated box 2 fused to lacZ (box1,2mut−lacZ) relative to those with box1,2,3−lacZ and box1−lacZ under P_7, limitation (LP) was a surprising result. It indicates that PhoB does interact with the mutant Pho box 2, although with a lower affinity than it does with the wild-type sequence, and decreases (but does not cancel) lacZ expression induced by PhoB−box1.

In fact, the β-galactosidase activity in the wild-type strain transformed with the plasmid carrying box1,2mut−lacZ was higher than that in cells harboring box1,2−lacZ but did not reach the levels found in wild-type or box1−lacZ cells.

We also investigated the effect of the mutated Pho box 2 on lacZ expression driven by the 234-bp full-length phoBR regulatory region in cells grown under low P_7, (LP) conditions. Interestingly, in the wild-type strain carrying box1,2mut,3−lacZ, the β-galactosidase activity level was slightly lower than that in cells carrying the construct box1,2,3−lacZ (Fig. 5a), showing that PhoB interacted with the mutated box 2 in vivo. However, the fact that higher β-galactosidase activity was observed in wild-type cells carrying box1,2mut,3−lacZ than in those harboring box1,2mut−lacZ suggests that PhoB binds to box 3 and partially relieves the transcriptional repression imposed by PhoB−box2mut (Fig. 5a). It is worthy of note that the interaction of PhoB with box 3 in this particular case seems to have been hindered by the abnormal associations of the PhoB−box2mut construct, which is in agreement with in vitro results described before (Fig. 2c).

In contrast, purified PhoB did not bind to the mutant Pho box 2 in vitro, as observed by the footprint assay (Fig. 2c, right). This provides evidence for a distinct binding pattern of PhoB to the regulatory region of phoBR in vivo, which might depend on the assistance of unknown cellular factors. Taken together, these results suggest that in wild-type V. cholerae cells under P_7, limitation, PhoB−box2 imposes a strong repression on the transcriptional activity of phoBR promoter induced by PhoB−box1, which is attenuated by a mutation within Pho box 2 or reversed by the binding of PhoB to box 3. A particularly noteworthy aspect of this regulatory mechanism is the strong positive effect of PhoB−box3 on the transcription of phoBR in wild-type cells under P_7, limitation. Accordingly, in wild-type cells harboring the construct box1,2−lacZ the β-galactosidase activity was close to zero, but in those harboring the full-length wild-type phoBR promoter (wild-type and box1,2,3−lacZ) it reached its highest level (Fig. 5a), highlighting the essential role of Pho box 3 in the positive control of phoBR expression in vivo under P_7, limitation. The fact that similar β-galactosidase activity levels were found in wild-type cells transformed with plasmid carrying box1,2,3−lacZ or box1−lacZ under low P_7, conditions strongly indicates that in vivo PhoB−box3 overcomes the negative effect of PhoB−box2 on the transcription of the phoBR operon.

Another important feature of the system is the location of boxes 2 and 3 in an antisense orientation with respect to the phoBR operon. The presence of PhoB binding sites in different strands of the regulatory region supports the hypothesis that PhoB exerts distinct functions in the control of phoBR expression in V. cholerae. As discussed previously, the binding of PhoP to the noncoding strand of tagAB and tagDEF in B.
expression of the operon in vivo. The regulatory role of each of the three Pho boxes (Fig. 6). Only PhoA activity in cells grown under high (HP) and low (LP) Pi conditions. HPoBR cells and HPoBR cells transformed with the empty vector were used as negative controls, and the wild-type strain was used as a positive control. Ø, no plasmid; vector, empty plasmid; 1,2,3, boxes 1, 2, and 3 fused to phoBR; 1,2 M3, box 1, mutated box 2, and box 3 fused to phoBR; 1,2 M, box 1 and mutated box 2 fused to phoBR; 1,2, boxes 1 and 2 fused to phoBR, 1, box 1 fused to phoBR.

PhoA activity in the V. cholerae ΔphoBR mutant complemented with low-copy-number plasmids containing the phoBR operon under the control of different versions of its regulatory region. PhoA activity was measured in cells grown under high (HP) and low (LP) Pi conditions. HPoBR cells and HPoBR cells transformed with the empty vector were used as negative controls, and the wild-type strain was used as a positive control. Ø, no plasmid; vector, empty plasmid; 1,2,3, boxes 1, 2, and 3 fused to phoBR; 1,2 M3, box 1, mutated box 2, and box 3 fused to phoBR; 1,2 M, box 1 and mutated box 2 fused to phoBR; 1,2, boxes 1 and 2 fused to phoBR, 1, box 1 fused to phoBR.

As expected, ΔphoBR mutant cells harboring plasmids with different fragments of the phoBR regulatory region upstream of lacZ presented very low β-galactosidase activities that were independent of Pi, levels (Fig. 5b). Interestingly, in ΔphoBR cells carrying box1,2,3–lacZ, independently of the extracellular Pi concentration the full-length promoter activity level was low and similar to that found in the wild type and box1,2,3–lacZ under Pi, abundance (Fig. 5b). These results strengthen the hypothesis that unknown activators with binding sites within the phoBR regulatory region play roles in its transcriptional control.

Pho regulon expression in V. cholerae requires a sequence containing Pho boxes 1, 2, and 3 immediately upstream of the translation start codon of the phoB gene. To better understand the regulatory role of each of the three phoBR Pho boxes in the expression of the operon in vivo, ΔphoBR mutant cells were complemented with low-copy pWKS30 derivatives containing phoBR genes under the control of different fragments of its regulatory region (Table 1). Cells were grown under high (HP) and low (LP) Pi levels for 16 h, and the expression of the Pho regulon genes was indirectly monitored by the activity of alkaline phosphatase (PhoA), the natural reporter of the regulon (Fig. 6). Only ΔphoBR cells complemented with box1,2,3–phoBR expressed PhoA at a level similar to that of the wild-type strain under Pi limitation. This observation corroborates the hypothesis that the three Pho boxes are required simultaneously for the efficient expression of the Pho regulon (Fig. 5a and 6).

These findings, together with those of the lacZ fusion assays (Fig. 5), point to a complex mode of regulation of the V. cholerae phoBR operon. β-Galactosidase activity assays revealed that the phoBR promoter region containing only Pho box 1 was transcriptionally activated by PhoB under low Pi conditions in wild-type cells. However, this version of the phoBR promoter was not active in the phoB-deficient background, since no PhoA expression was detected under low Pi conditions. An obvious explanation is that ΔphoBR is a PhoB-deficient strain and a basal level of the protein is required to induce phoBR expression to a detectable level. On the other hand, ΔphoBR mutant cells complemented with the full phoBR regulatory region (box1,2,3–phoBR) presented PhoA activity under low Pi, suggesting phoBR expression. The slightly higher PhoA activity in ΔphoBR and box1,2,3–phoBR cells relative to the wild type might be due to the vector used for the complementation assays, which is present in 6 to 8 copies per cell (49). Promoter versions with box1,2mut,3 or box1,2mut did not drive the transcription of phoBR in ΔphoBR cells under Pi limitation, since no PhoA expression was observed. Nevertheless, they activated lacZ transcription in wild-type cells (Fig. 5a). These findings indicate that the expression of Pho regulon genes in vivo depends on a basal level of PhoB in the cells which, in turn, relies on the presence of a full-length regulatory region of phoBR.

Concluding remarks. V. cholerae Pho regulon members have important roles, not only in the response to environmental phosphate limitation but also in pathogenesis (9, 10, 36, 37, 43, 47, 48). Therefore, it was not surprising to discover a tight control over the expression of phoBR and, consequently, of PhoB-dependent genes.

Data presented here indicate that the expression of the V. cholerae phoBR operon depends on a peculiar binding pattern of PhoB to three Pho boxes. Moreover, the interaction of PhoB with distinct boxes either activates or represses phoBR expression. Based on these observations and on the set of results of the present study, we propose a hypothesis to explain the molecular events involved in V. cholerae phoBR autoregulation. Under Pi, limiting conditions, basally expressed PhoB, activated by phosphorylation, binds with high affinity to Pho box 1, which is closest to the σ70 binding site on the phoBR regulatory region. PhoB–box1 complex would cause a considerable bending in the DNA molecule, thus enabling interaction between PhoB and the RNA polymerase to activate transcription, as previously observed in E. coli (24). As the PhoB cellular level increases by positive autoregulation, the protein would bind to the lower affinity box 2, upstream of box 1, in the antisense promoter strand. Operon fusion assays showed virtually no lacZ transcription from the regulatory sequence carrying only boxes 1 and 2 in wild-type cells. Thus, we suggest that the binding of PhoB to box 2 would introduce changes in the spatial organization of the promoter sequence, reducing the interaction between PhoB–box1 and the RNA polymerase σ70. More precisely, PhoB–box2 (in the antisense strand) might bend the DNA in the opposite orientation, affecting negatively the interaction of PhoB–box1–RNA polymerase, thereby reducing the transcriptional rate of phoBR.

As shown above, the binding of PhoB to box 3 seems to require PhoB–box2, which is not surprising, since box 3 contains more mismatches relative to the consensus sequence than...
box 1 and box 2 and, from the three loci, is the one with the lowest binding affinity for PhoB. Pho box 3 is in the antisense strand of the phoBR promoter, thus the complex PhoB–box3 might further alter the structure of the regulatory sequence and the spatial arrangement of the bound molecules with respect to each other. This might bring PhoB–box 1 into close proximity to the RNA polymerase, thus activating the transcription of phoBR. Rounds of the binding/unbinding of PhoB to these boxes and the resulting changes in structural features of the regulatory region ultimately would keep the transcription under tight control and the expression levels of the operon adjusted to the cell needs. Thus, PhoB exerts a dual regulatory function, as an activator and repressor, by binding to distinct Pho boxes in a sequential mode and with different affinities, enabling V. cholerae cells to fine-tune the expression of the phoBR operon under Pi limitation. Under Pi abundance, the V. cholerae phoBR operon does seem to be expressed, but at a lower level, apparently by the interaction of unknown activators to its regulatory region. This would ensure a basal level of PhoB, which is required not only for the Pi-independent roles that PhoB plays in the cells but also for the expression of the Pho regulon when V. cholerae faces Pi-limiting conditions.

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