

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 *phoB* 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 (P_i) limitation. When a fragment, including only boxes 1 and 2, was fused to *lacZ*, expression was no longer induced by low P_i , 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* $\Delta phoBR$ cells. This was observed under P_i abundance, when *phoBR* was expressed at a basal level and, also in low P_i conditions, when Pho regulon genes were fully expressed. Thus, under P_i 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 (P_i), or coenzymes.

In many bacterial species, phosphorus-containing compound uptake and metabolism depend on the extracellular P_i concentration. Under P_i 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 P_i 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 P_i to its cognate response regulator, PhoB (26). Phosphorylated PhoB (PhoB~ P_i) 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)TCAT A(AT)A(AT) CTGTCA(TC), consists of two 7-bp direct repeats separated by a conserved

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TABLE 1. Bacterial strains and plasmids used in this study^a

Strain or plasmid	Description	Source or reference
<i>E. coli</i> strains		
DH5 α	F ⁻ <i>endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 recA1 gyrA</i> (Nal ^r) <i>relA1</i> Δ (<i>lacZYA argF</i>) <i>U169</i> ϕ 80 <i>lacZ</i> Δ M15	38
BL21(DE3)	<i>hsdS gal</i> (λ CIts857 <i>ind1 Sam7 nin5 lacUV5-T7</i> gene 1)	42
<i>V. cholerae</i> strains		
569BSR	Classical Inaba, Sm ^r , wild-type <i>V. cholerae</i>	47
WK3	Δ <i>phoBR</i> mutant of 569BSR; Sm ^r Km ^r	47
Plasmids		
pGEX4T-3	High-copy vector containing an MCS preceded by GST coding region; Ap ^r	GE Healthcare
pGEX <i>pho</i> WT	0.7-kbp 569BSR <i>phoB</i> ORF inserted into BamHI/XhoI pGEX4T-3 restriction sites, fused 5' to GST coding region; Ap ^r	This work
pIC552	Low-copy vector containing <i>lacZ</i> preceded by an MCS to insertion of a promoter region; Ap ^r	23
pIC234	pIC552 containing a region of 234 bp upstream of <i>phoBR</i> putative transcription start site regulating <i>lacZ</i> ; Ap ^r	10
pIC86	pIC552 containing a region of 86 bp upstream of <i>phoBR</i> putative transcription start site regulating <i>lacZ</i> ; Ap ^r	This work
pIC62	pIC552 containing a region of 62 bp upstream of <i>phoBR</i> putative transcription start site regulating <i>lacZ</i> ; Ap ^r	This work
pIC234mut	pIC552 containing the mutated region of 234 bp upstream of <i>phoBR</i> putative transcription start site regulating <i>lacZ</i> ; Ap ^r	This work
pIC86mut	pIC552 containing the mutated region of 86 bp upstream of <i>phoBR</i> putative transcription start site regulating <i>lacZ</i> ; Ap ^r	This work
pWKS30	Low-copy vector; Ap ^r	49
pGEM-T Easy	High-copy easy cloning vector; Ap ^r	Promega
pWK5	High-copy plasmid; contains <i>phoBR</i> operon, including entire regulatory region; Ap ^r	47
pWK20	3 kbp pWK5 PstI fragment inserted into PstI pWKS30 restriction site; contains <i>phoBR</i> coding region plus 234-bp promoter; Ap ^r	This work
pCG7	pWK5 containing a SacII restriction site in the center of <i>phoBR</i> regulatory region; Ap ^r	This work
pCG8	3-kbp pCG7 PstI fragment inserted into PstI pWKS30 restriction site; contains <i>phoBR</i> coding region plus 234-bp mutated promoter; Ap ^r	This work
pLC1	pWK5 reverse amplified, digested with BglII, and linked; contains <i>phoBR</i> plus 62-bp promoter; Ap ^r	This work
pLC2	pWK5 reverse amplified, digested with BglII, and linked; contains <i>phoBR</i> plus 86-bp promoter; Ap ^r	This work
pLC3	2.3-kbp pLC1 BglII/PstI fragment, inserted into BamHI/PstI pWKS30 restriction sites; contains <i>phoBR</i> plus 62-bp promoter; Ap ^r	This work
pLC4	2.3-kbp pLC2 BglII/PstI fragment, inserted into BamHI/PstI pWKS30 restriction sites; contains <i>phoBR</i> plus 86-bp promoter; Ap ^r	This work
pLC5	2.3-kbp fragment of pCG7 amplified and cloned into vector pGEM-T Easy; contains <i>phoBR</i> plus 86-bp mutated promoter; Ap ^r	This work
pLC6	2.3-kbp pLC5 NotI fragment, inserted into NotI pWKS30 restriction site; contains <i>phoBR</i> plus 86-bp mutated promoter; Ap ^r	This work

^a Sm, streptomycin; Km, kanamycin; Ap, ampicillin; Cm, chloramphenicol; MCS, multiple-cloning site; ORF, open reading frame.

4-bp AT rich spacer, where PhoB binds as a head-to-tail dimer and interacts with the RNA polymerase σ^{70} 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 appropriate gene expression (21, 35). The *E. coli* Pho regulon consists of about 40 genes and includes the *phoBR* operon (18, 50), which is transcriptionally autoregulated via the direct binding of PhoB to a single Pho box at –35, 10 bp upstream of the putative –10 region (28, 45).

In a previous work, we have shown that in *V. cholerae* *phoR* and *phoB* genes constitute an operon whose regulatory region contains a putative Pho box sequence located at –35, as in *E. coli* (47). Moreover, mutation in *phoBR* eliminated the expression of Pho regulon genes in strains of *V. cholerae* O1 under P_i limitation. Due to the involvement of the *V. cholerae* O1 *phoBR* operon in many physiological processes and in viru-

lence (9, 36, 37, 43, 47), we decided to further investigate the 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 abundance.

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

propagated in LB agar or LB for liquid cultures (39). The minimal medium TG (5) was used for bacterial growth under high (6.5 mM; TGHP) or low (65 μ M; TGLP) P_i concentration. When required, 100 μ g/ml ampicillin, 100 μ g/ml streptomycin, and/or 50 μ g/ml kanamycin was added to the media. Cultures were grown at 37°C in a shaking water bath at 200 rpm or in an incubator.

Genetic methods and cloning procedures. All genetic manipulations were carried out in *E. coli* DH5 α (38). *V. cholerae* chromosomal DNA was purified as described previously (1) from overnight cells cultured in LB. Standard methods were used for minipreparations of plasmids, restriction enzyme digestions, and ligations (39). DNA fragments, unless specified, were purified from agarose gels with Illustra GFX PCR DNA (GE Healthcare) in accordance with the supplier's recommendations. The preparation of *E. coli* competent cells and electroporation were carried out as described previously (39), whereas *V. cholerae* competent cells were prepared and electroporated as described previously (29, 47). PCR amplifications were performed in an automatic thermal cycler (Ominigene) using standard conditions (14).

Different fragments of the putative *phoBR* regulatory region were cloned into pIC552 to generate *lacZ* fusions. To this end, pWK5 (47), a plasmid containing the *phoBR* operon and an ~400-bp-long sequence upstream of *phoB*, was used as a template for PCR. Plasmid pIC234 containing the 234-bp *phoBR* full-length regulatory region upstream of *lacZ* was obtained in a previous work (10). Fragments 86 and 62 bp long of the *phoBR* regulatory region were obtained by PCR amplification with oligonucleotides pIC170F (5'GCGAGATCTGCTGAACC TTA3') and PhoBXhoI (5'TTCTTCTCGAGTGTTTTTCACCAACGCCA3') as well as Met2E (5'CGGGCCATGGTTTGCAAAGGTACAAAAG3') and PhoBXhoI, respectively. Purified PCR products were digested with NcoI and XhoI and cloned upstream of the *lacZ* gene into pIC552 that had been opened with the same endonucleases (23), generating pIC86 and pIC62, respectively (Table 1).

To introduce a mutation at approximately –60 of the putative translation start site of *phoB* within Pho box 2, pWK5 was used as a template in a reverse PCR along with oligonucleotides CPMut1 (5'TCACCCGCGGATGAAAAATTGTT GC3') and CPMut2 (5'ATTACCGCGGTTCAAGCCGAAATTC3'), containing sites for the restriction enzyme SacII at their 5' ends. Pfx Triple Master (Eppendorf) was used according to the supplier's instructions, and the PCR product was digested with SacII and ligated. In this process, the AT-rich motif TTAT in the wild-type sequence was replaced by GCGG. The recombinant plasmid, namely, pCG7, was used as the template for subsequent amplifications. Different segments of the *phoBR* regulatory region containing the mutated sequence were cloned in pIC552 upstream of *lacZ*. To this end, mutated fragments of 234 and 86 bp were PCR amplified using the primers pairs PhoBNcoI (5'CCCCCATGGTTTAAACCACATGTGTTG3') and PhoBXhoI as well as P170Fmut (5'GCGAGATCTGCCTGAACCGCG3') and PhoBXhoI, respectively. Purified fragments were digested with NcoI and XhoI, cloned into pIC552, and opened with these enzymes, generating pIC234mut and pIC86mut (Table 1).

For the complementation analysis of the *phoBR* mutant strain WK3 (Δ *phoBR*) (Table 1), low-copy-number plasmids containing the *phoBR* operon under the control of different fragments of its own regulatory region were obtained as described below. Initially, pWK5 was digested with PstI to remove a 3-kbp fragment containing *phoBR* and the full-length regulatory region. The low-copy-number plasmid pWKS30 (49) was opened with the same enzyme and ligated to the *phoBR* PstI fragment, generating plasmid pWK20. To obtain plasmids containing the *phoBR* operon under the control of shorter fragments of its wild-type regulatory region, pWK5 was subjected to reverse PCR using Pfx Triple Master. P50F (5'GCGAGATCTGGTCACAAAAGT3') and V50RA (5'GCGAGATCT AAAGTGGTCACCAAG3') or P170F and V50RA were used to amplify fragments containing 62 or 86 bp, respectively. PCR products were digested with BglII and ligated, generating pLC1 and pLC2, respectively. Both plasmids then were double digested with PstI and BglII, and fragments of approximately 2.3 kbp were cloned into pWKS30 digested with the same enzymes, creating pLC3 (62 bp upstream of *phoBR*) and pLC4 (86 bp upstream of *phoBR*). To generate a plasmid with the full-length regulatory region mutated within Pho box 2, pCG7 was digested with PstI to remove a 3-kbp fragment that subsequently was cloned into the PstI site of pWKS30, generating plasmid pCG8. Plasmid with a version of the promoter containing Pho box 1 and mutated box 2 (2mut) was obtained using pCG7 as the template for PCR with Pfx Triple Master and the oligonucleotides LIV1 (5'AATAATTTGCGCTGAACCGC3') and P50R (5'TCTAGA GGATCCCGGGTA3'). The 2.3-kbp product (86 bp upstream of *phoBR*) was purified and cloned into pGEM-T Easy (Promega), resulting in plasmid pLC5. This in turn was digested with NotI to release the 2.3-kbp fragment that was inserted into the NotI site of pWKS30, generating plasmid pLC6.

For the expression of the 569BSR *phoB* gene in *E. coli*, its coding sequence was

amplified using oligonucleotide primers PhoBini (5'GGCGCGGATCCAGAA GGATTCTGGTTGTGAAG3') and CterXhoI (5'GCGCCGCTCGAGTTAG GCTTTGG3'), with 569BSR genomic DNA as the template. The 0.7-kbp fragment was digested with BamHI and XhoI and cloned into pGEX4T-3 (GE Healthcare) digested with the same enzymes, generating pGEX*phoWT*, which contains the glutathione S-transferase (GST) coding sequence 5' of *phoB*. All constructions were screened and confirmed by PCR and sequencing (ABI PRISM BigDye Terminator cycle sequencing; Applied Biosystems).

Overexpression of *Vibrio cholerae* O1 *phoB* and PhoB purification. Strain BL21(DE3) (Table 1) harboring pGEX*phoWT* was inoculated into 1 liter of LB-ampicillin and grown with aeration at 37°C until an optical density at 600 nm (OD₆₀₀) of 0.7. Isopropyl- β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the culture was further incubated under the same conditions for 4 h. Cells were collected by centrifugation and resuspended in phosphate-buffered saline (PBS) containing 1% (wt/vol) Triton X-100 at a proportion of 5 ml/g (wet weight) of cells. Aliquots of 1.25 mg lysozyme/g of cells were added to the suspension before sonication (Sonicator W-380; Heat Systems Ultrasonics, Inc.) until complete lysis. The lysate was centrifuged at 11,000 \times g for 10 min at 4°C, and the soluble fraction, containing the major amount of fusion protein, was incubated with glutathione Sepharose resin (GE Healthcare) according to the manufacturer's instructions. The GST-PhoB fusion protein was purified and then cleaved by thrombin (GE Healthcare). The purity and concentration of the recombinant PhoB were 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 *phoBR*. A search for Pho box-like sequences within the 234-bp *phoBR* regulatory region was performed in both sense and antisense orientations using the GLAM2SCAN tool (8), part of the MEME suite (2), based on the consensus *E. coli* Pho box motif (24), which was constructed with the GLAM2 tool.

EMSA. 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'CCAAGGTACAAAAGTGCATAA A3') and cx1rev (5'TTTATGACACTTTTGTGACCTTGG3'), cx2fwd (5'TGA ACCTTATATGAAAATTTGTTG3') and cx2rev (5'CAACAATTTTCATAT AAGGTTCA3'), and cx3fwd (5'AGGTTTGGATATATGAATTTCCGC3') and cx3rev (5'GGCGAAAATTCATATATCCAAACT3'). 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, 35°C for 10 min, and 25°C for 5 min. The PCR products and the annealed double-stranded oligonucleotides were purified from 15% polyacrylamide gels (39), quantified, and radiolabeled at the 5' end with [γ -³²P]ATP using T4 polynucleotide 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 *phoBR* regulatory region contained 50 fmol of probe and increasing concentrations of PhoB (from 0 to 32 μ M) in binding buffer (20 mM Tris-HCl, pH 7.0; 50 mM NaCl; 1 mM dithiothreitol [DTT]; 10 mM MgCl₂; 100 μ g/ml bovine serum albumin [BSA]; 0.5 μ g/ml salmon sperm DNA) in a total volume of 10 μ l. Competition assays were performed using 50 fmol of radiolabeled promoter fragment, PhoB at 32 μ M, 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, 0.5, 2.5, 5, 10, 20, 30, and 37 μ M). 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 μ M). For competition assays, 50, 250, and 500 fmol of unlabeled oligonucleotides was added to reaction mixtures containing 30 μ M 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 μ M (control). Another set of reaction mixtures contained PhoB at 30 μ M, 50 fmol of labeled Pho box 1, and a total of 500 fmol of unlabeled 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 \times

Tris-acetate-ETDA buffer (39). Gels were exposed to a PhosphorImager screen (Molecular Devices) cassette sealed against light for 24 h.

DNase I footprinting. The oligonucleotide PhoBXhoI was radiolabeled at the 5' end using [γ - 32 P]ATP and T4 polynucleotide kinase (39). After labeling, the oligonucleotide was precipitated and resuspended in diethyl pyrocarbonate (DEPC)-treated water. The 32 P oligonucleotide PhoBXhoI and nonlabeled PhoBcoI oligonucleotide were used as primers to amplify the 234-bp sequence upstream of *phoBR* using pWK5 (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 μ l of sequencing buffer (T7 sequencing kit; GE Healthcare) and subjected to gel electrophoresis alongside DNase I digestions products (30).

Purified *V. cholerae* PhoB (0, 0.5, 2.5, and 10 μ M) was incubated with 200 fmol of wild-type or mutated 234-bp DNA probe in binding buffer in a total volume of 49 μ l. Reaction mixtures were incubated for 30 min at room temperature, and then 0.01 U of DNase I was added per tube for probe digestion. Reaction tubes were left at room temperature for 1 min, after which 140 μ l 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 μ l of sequencing buffer. Products of DNase I digestion, as well as G+A sequencing, were subjected to electrophoresis on a 6% polyacrylamide gel under denaturing conditions. The gel was prerun at a constant voltage of 1,400 to 1,700 V and 40 W until the temperature reached 50°C, when samples were applied. After being run, the gel was dried at 80°C for 1 h and exposed to a PhosphorImager screen cassette sealed to light for 24 h.

Enzymatic assays. For promoter activity assay, *V. cholerae* wild-type (569BSR) and Δ *phoBR* mutant (WK3) strains containing plasmid pIC552, pIC234, pIC86, pIC62, pIC234mut, or pIC86mut were grown in LB medium until an OD₆₀₀ of 0.5. Cells were collected by centrifugation, resuspended in the same volume of TGHP (P_i abundance) or TGLP (P_i 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 regulon expression, was determined as previously described (47) in the same cells. Activities of both enzymes were expressed in U/ μ g of protein (22).

For complementation assays, *V. cholerae* Δ *phoBR* 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 32 P-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 μ M, 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

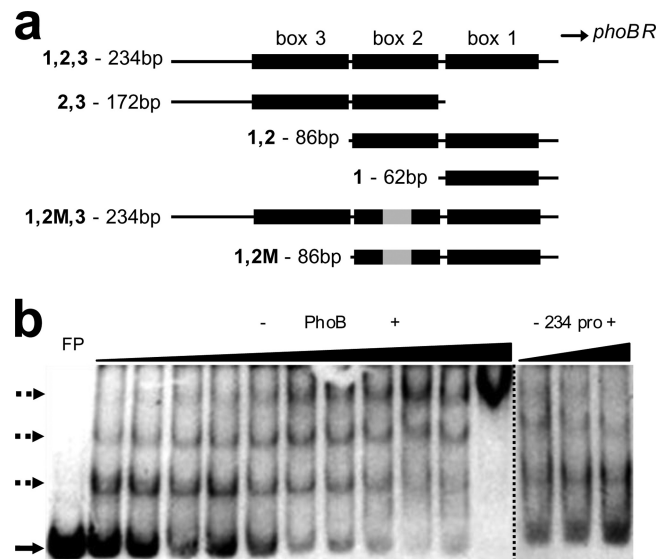


FIG. 1. (a) Scheme of *phoBR* regulatory region fragments used in this work. Pho boxes are represented by black rectangles; mutation in box 2 is represented by a gray rectangle. (b) EMSA pattern of the *phoBR* 234-bp regulatory region fragment radiolabeled with 32 P by PhoB binding. In the left panel, 50 fmol of the fragment was incubated with increasing concentrations of purified PhoB (1.5, 2, 2.5, 3, 3.5, 4, 8, 12, 16, 20, and 32 μ M). In the first lane, the FP (free probe) position is indicated by a solid arrow; dotted arrows point to three distinct shifted bands. In the right panel is the result of the competition assay using 50 fmol of radiolabeled 234-bp promoter fragment and PhoB at 32 μ M against 50, 250, and 500 fmol of unlabeled probe.

grown under P_i 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-

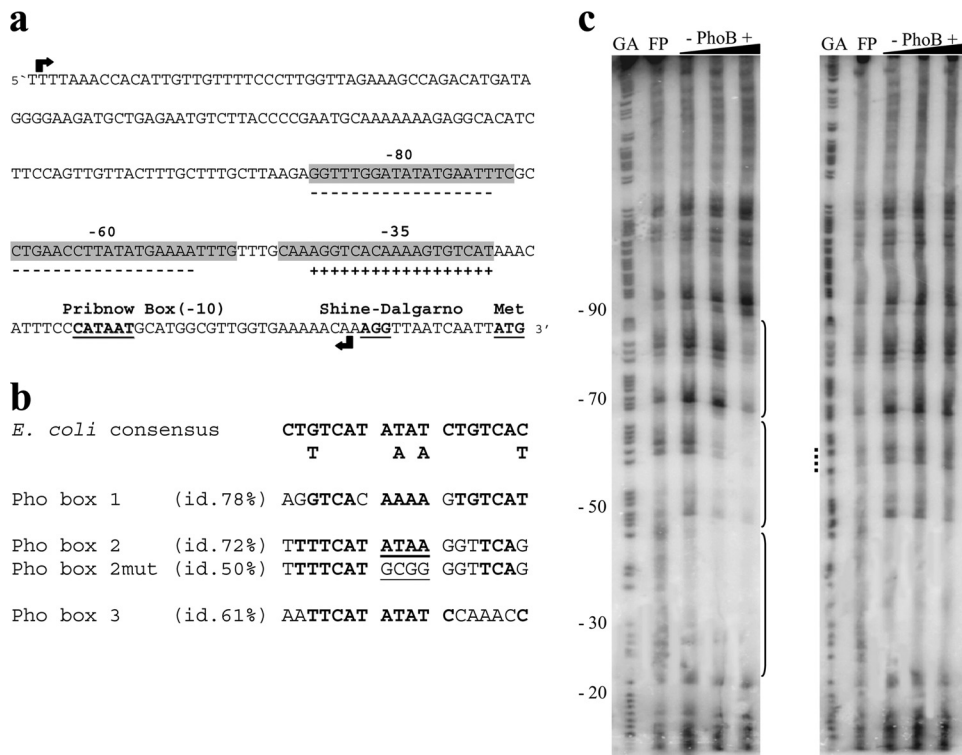


FIG. 2. Mapping of Pho boxes in the *phoBR* upstream region of *V. cholerae*. (a) Diagram of the *phoBR* regulatory region, with the Pho boxes, identified by similarity search with GLAM2SCAN, and gaps. Sequences corresponding to footprint gaps are shown in gray; sequences identified as Pho boxes are marked with a plus (sense orientation) or minus (antisense orientation). Black arrows indicate the limits of the 234-bp sequence. (b) Pho box sequences in the *phoBR* regulatory region and their identities to the *E. coli* consensus Pho box and the box 2 mutated (box 2mut) version. (c) Results of DNase I protection assays of PhoB binding sites (brackets) in the *phoBR* regulatory region. Two hundred fmol of the radiolabeled wild-type 234-bp sequence (left) and 234-bp box 2 mutated version (right) were independently incubated with increasing concentrations of PhoB (0.5, 2.5, and 10 μ M) and digested with DNase I. Digestion products were size fractionated by denaturing gel electrophoresis. GA is the sequencing ladder. The dotted line in panel c marks the positions of the mutated bases in Pho box 2mut. FP, free probe.

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 *pstS* (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).

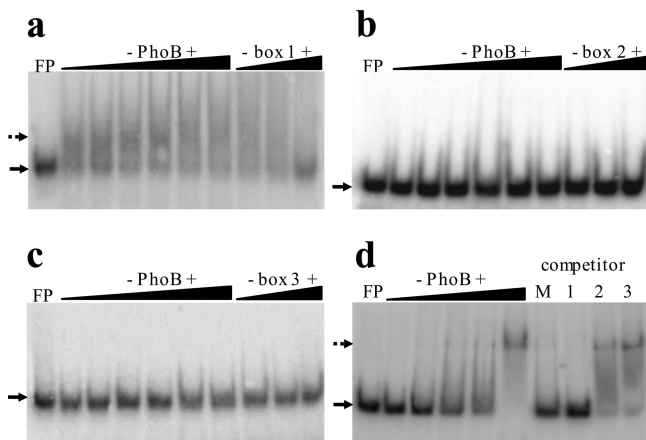


FIG. 3. Binding of PhoB to individual Pho boxes in the *phoBR* regulatory region. Fifty fmol of radiolabeled double-stranded oligonucleotides corresponding to each one of the three Pho boxes was used in EMSAs with increasing PhoB concentrations. The binding patterns (dotted arrows) of Pho box 1 (a), Pho box 2 (b), and Pho box 3 (c) to PhoB at 2, 5, 10, 20, 30, and 37 μ M are presented. Competition assays were carried out with 37 μ M PhoB plus 50, 250, and 500 fmol of competing nonlabeled oligonucleotides. (d) The labeled oligonucleotide corresponding to Pho box 1 was incubated with increasing PhoB concentrations (0.5, 2, 5, 10, and 30 μ M). Competition assay was performed with PhoB at 30 μ M against 500 fmol of competing nonlabeled Pho boxes containing fragments. Lane M, equimolar mixture of Pho boxes 1, 2, and 3 (a total of 500 fmol); lane 1, 500 fmol of Pho box 1; lane 2, 500 fmol of Pho box 2; lane 3, 500 fmol of Pho box 3. FP, free probe (solid arrows).

Therefore, the binding of PhoB to these sequences under P_i 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 μ M), 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 μ M) (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 *in vitro*.

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).

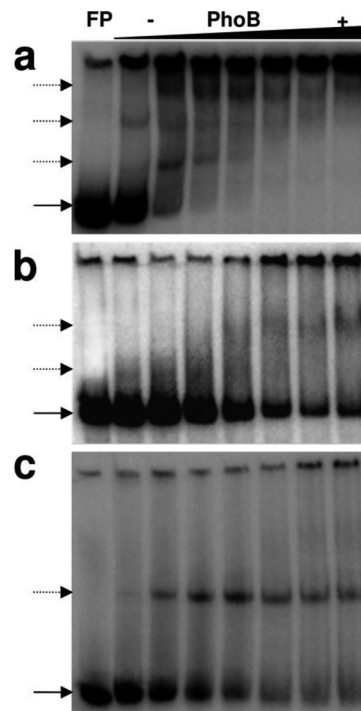


FIG. 4. Binding patterns of PhoB to three distinct *phoBR* promoter fragments. Fifty fmol of labeled double-stranded probes were used with increasing PhoB concentrations (0, 0.5, 2.5, 5, 10, 20, 30, and 37 μ M). Binding patterns of probes containing boxes 1, 2, and 3 (a), boxes 2 and 3 (b), or box 1 alone (c) are shown. In the first lane, the free probe (FP) position is indicated by a solid arrow; dotted arrows point to shifted bands.

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 μ M, 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 *phoBR* 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 P_i limitation (TGLP medium) or P_i abundance (TGHP medium) for 16 h, when the expression of both the *phoBR*

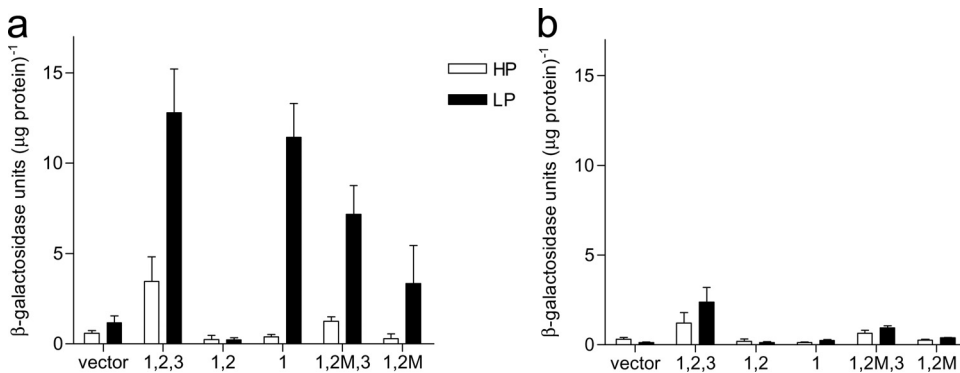


FIG. 5. β -Galactosidase activity levels in the wild-type (a) and $\Delta phoBR$ mutant cells (b) harboring vectors with different versions of the *phoBR* regulatory region fused to *lacZ*. Vector, promoterless *lacZ*; 1,2,3, boxes 1, 2, and 3 fused to *lacZ*; 1,2, boxes 1 and 2 fused to *lacZ*; 1, box 1 fused to *lacZ*; 1,2 M,3, box 1, mutated box 2, and box 3 fused to *lacZ*; 1,2 M, box 1 and mutated box 2 fused to *lacZ*. Strains were grown under high (HP) and low (LP) phosphate conditions for 16 h.

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_i limitation (LP), harboring either boxes 1, 2, and 3 fused to *lacZ* (box1,2,3~*lacZ*) or box1~*lacZ* (Fig. 5a). Under P_i 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 Pho box 1 is strictly dependent on P_i 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_i rich medium, in agreement with previous findings (9, 10, 36, 47, 48).

Surprisingly, wild-type cells carrying box1,2~*lacZ* cultured either in P_i limitation (LP) or P_i 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_i 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 of 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_i 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_i (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 inter-

acted 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_i 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_i limitation. Accordingly, in wild-type cells carrying 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_i 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_i 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.*

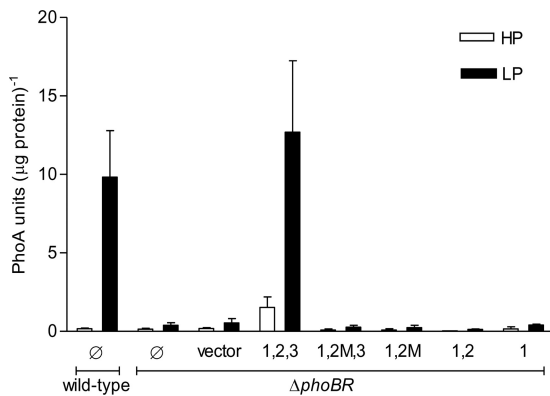


FIG. 6. PhoA activity levels in the *V. cholerae* $\Delta phoBR$ mutant complemented with low-copy-number plasmids containing the *phoBR* operon under the control of different versions of its regulatory region. PhoA activity was measured in cells grown under high (HP) and low (LP) P_i conditions. $\Delta phoBR$ cells and $\Delta phoBR$ cells transformed with the empty vector were used as negative controls, and the wild-type strain was used as a positive control. \emptyset , no plasmid; vector, empty plasmid; 1,2,3, boxes 1, 2, and 3 fused to *phoBR*; 1,2 M,3, 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*.

subtilis is critical for its negative role in the control of the expression of these genes (21). Thus, the antisense location of Pho box 2 could partially explain the repression of *phoBR* transcription mediated by PhoB~box2 (Fig. 5a). However, Pho box 3 (at -80), immediately upstream of box 2 and also on the noncoding strand of *phoBR*, on the contrary, cancelled PhoB~box2-mediated repression *in vivo*.

As expected, $\Delta phoBR$ 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 P_i levels (Fig. 5b). Interestingly, in $\Delta phoBR$ cells carrying box1,2,3~*lacZ*, independently of the extracellular P_i concentration the full-length promoter activity level was low and similar to that found in the wild type and box1,2,3~*lacZ* under P_i 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*, $\Delta phoBR$ 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) P_i 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 $\Delta phoBR$ cells complemented with box1,2,3~*phoBR* expressed PhoA at a level similar to that of the wild-type strain under P_i 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 P_i 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 P_i conditions. An obvious explanation is that $\Delta phoBR$ 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, $\Delta phoBR$ mutant cells complemented with the full *phoBR* regulatory region (box1,2,3~*phoBR*) presented PhoA activity under low P_i , suggesting *phoBR* expression. The slightly higher PhoA activity in $\Delta phoBR$ 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 $\Delta phoBR$ cells under P_i 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 very 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 P_i limiting conditions, basally expressed PhoB, activated by phosphorylation, binds with high affinity to Pho box 1, which is closest to the σ^{70} 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 σ^{70} . More precisely, PhoB~box 2 (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 P_i limitation. Under P_i 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 P_i -independent roles that PhoBR plays in the cells but also for the expression of the Pho regulon when *V. cholerae* faces P_i -limiting conditions.

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