A role for the PhoBR regulatory system homologue in the \textit{Vibrio cholerae} phosphate-limitation response and intestinal colonization

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To survive and multiply in different environments, \textit{Vibrio cholerae} has to co-ordinately regulate the expression of genes involved in adaptive responses. In many pathogens, adaptive responses, including pathogenic responses, are regulated by two-component regulator (TCR) systems. It is likely that members of a TCR family play a role in the regulation of processes involved in intestinal colonization, and therefore pathogenesis, in \textit{V. cholerae}. We have identified and characterized a TCR system of \textit{V. cholerae}: this system is a homologue of \textit{Escherichia coli} PhoBR. The presence of a putative Pho box suggests that the \textit{V. cholerae} phoBR operon is regulated by inorganic phosphate levels. The phoR and phoB genes are organized the same way as in \textit{E. coli}. Mutation of the \textit{V. cholerae} phoB gene affected the expression of the putative Pho regulon, including PhoA, but did not affect the production of cholera toxin. \textit{V. cholerae} phoB mutants are less able to colonize rabbit intestine than wild-type \textit{V. cholerae}. The addition of inorganic phosphate at a high concentration to the inoculum only partially restored the ability of the mutants to colonize the intestine, suggesting that the \textit{V. cholerae} Pho regulon \textit{in vivo} may not be regulated by inorganic phosphate levels alone.

Keywords: \textit{Vibrio cholerae}, PhoBR, two-component, regulation, colonization

INTRODUCTION

\textit{Vibrio cholerae} O1 causes cholera, a severe diarrhoeal disease acquired by ingestion of the organisms in contaminated food or water. After surviving the gastric barrier, the bacteria enter the lumen of the gut, where they colonize the small intestinal epithelium and secrete cholera toxin (CT). CT acts on intestinal epithelial cells, causing an electrolyte imbalance accompanied by water loss, which, in severe cases, is followed by the collapse of the circulatory system and death (Bennish, 1994). To survive and multiply in different and changing environments such as the intestine, \textit{V. cholerae} regulates co-ordinately the expression of genes involved in particular adaptive responses. In \textit{V. cholerae}, ToxR regulates both directly and indirectly the expression of about 17 genes (Peterson & Mekalanos, 1988) including those encoding CT (Miller & Mekalanos, 1984), the colonization factor TCP (Taylor et al., 1987), accessory colonization factors (Peterson & Mekalanos, 1988) and outer-membrane proteins OmpT and OmpU (Miller & Mekalanos, 1988). ToxR regulation is part of a complex regulatory network (reviewed by Skorupski & Taylor, 1997) that involves ToxT (a member of the AraC regulatory family) and the global regulator cAMP-CRP. Although ToxR plays a central role in the control of virulence, not all putative virulence-determinant genes are under its control (Goldberg et al., 1990) and pathogenesis cannot be fully explained by known determinants (Levine & Tacket, 1994). In a variety of pathogens, adaptive responses, including pathogenic responses, are regulated by two-component regulator (TCR) systems (Miller et al., 1989). Until recently (Humphreys et al., 1995; Lee et al., 1998), no members of the TCR family had been
described in *V. cholerae* (Skorupski & Taylor, 1997). The *vieSAB* system was identified using an approach where the *vieB* gene was shown to be expressed in *vivo* (Lee et al., 1998). The histidine kinase (HK) VieS is most similar to BygS, whilst the response regulator (RR) components in the operon, VieA and VieB, are similar to CheY (Lee et al., 1998). Although the VieSAB system did not appear to play a role in intestinal colonization, it is likely that other TCRs may have a role in regulating the complex processes involved in intestinal colonization and pathogenesis.

Limitation of inorganic phosphate leads bacterial cells to synthesize a number of proteins, many being involved in the acquisition of phosphate (Wanner, 1996). Phosphate limitation can also play a role in microbial virulence. In *Pseudomonas aeruginosa*, the expression of an extracellular heat-labile haemolysin is induced under phosphate-limiting conditions and is required for virulence (Ostroff et al., 1989). In *Agrobacterium tumefaciens* (Aoyama et al., 1991; Winans, 1990) and *Salmonella typhimurium* (Libby et al., 1990), low phosphate levels induce synthesis of virulence factors. Furthermore, mutations in entero-invasive *Escherichia coli* genes involved in phosphate acquisition gave a hyper-invasive phenotype (Sinai & Bavoil, 1993), and in an *E. coli* strain pathogenic to pigs, mutation of a gene of the Pho regulon (Lee et al., 1989) produced an avirulent strain (Daigle et al., 1995).

The expression of proteins regulated by phosphate concentration requires PhoR and PhoB, members of a TCR system, where PhoR is the HK and PhoB the RR (Stock et al., 1989). The set of genes whose expression depends on PhoB constitutes the Pho regulon (Lee et al., 1989). Pho regulon promoters contain one or more copies of a conserved consensus sequence, the Pho box, that functions as a PhoB-binding site (Makino et al., 1986a).

In this report, we describe the cloning and sequencing of the *V. cholerae* *phoR* and *phoB* genes (designated *phoR*<sup>Vc</sup> and *phoB*<sup>Vc</sup>, respectively). Thus, this is the first description of a member of the OmpR subfamily of TCRs in *V. cholerae*, as ToxR's similarity with OmpR is limited to the DNA-binding motif (Miller et al., 1987) and the proteins are otherwise structurally and functionally different. Our analysis of *phoB*<sup>Vc</sup> mutants indicates that *V. cholerae* responds to phosphate limitation in a way similar to *E. coli* (Wanner, 1996). In addition, mutation of the *phoB*<sup>Vc</sup> gene reduced colonization ability, suggesting a role for the Pho regulon in adaptation of *V. cholerae* to the intestinal environment.

**METHODS**

**Bacterial strains, media and plasmids.** Bacterial strains and plasmids used in this work are shown in Table 1. Strains were routinely grown at 37 °C in Luria–Bertani broth (LB; Sambrook et al., 1989) or on solid medium (LA; 1·5% agar in LB). For growth under known inorganic phosphate concentrations, a medium containing a mix of mineral salts buffered with Tris/HCl (pH 6·5 or pH 8·0) supplemented with 0·2% glucose and 0·01 mM thiamin was used (TG medium; Ehols et al., 1961). For high or low phosphate conditions, TG was supplemented with KH<sub>2</sub>P<sub>O</sub><sub>4</sub> at final concentrations of 6·5 mM (TGHP) or 65 µM (TGLP), respectively. When amino acids were required, a mix containing asparagine, serine and glutamate was added to give a final concentration of 0·03% of each amino acid. Where necessary, antibiotics were added at the following concentrations: ampicillin, 100 µg ml<sup>−1</sup>; chloramphenicol, 20 µg ml<sup>−1</sup>; kanamycin, 50 µg ml<sup>−1</sup> (*E. coli*) or 25 µg ml<sup>−1</sup> (*V. cholerae*); streptomycin, 100 µg ml<sup>−1</sup>; tetracycline, 15 µg ml<sup>−1</sup> (*E. coli*) or 3 µg ml<sup>−1</sup> (*V. cholerae*). X-Gal (Boehringer Mannheim) for the selection of β-galactosidase-producing clones was used at 20 µg ml<sup>−1</sup).

**Genetic methods.** All genetic manipulations were carried out in *E. coli* DH<sub>5</sub>α, except those involving pGPT04 derivatives, which used *E. coli* strains SY327 βpir and SM10 βpir (Table 1). *V. cholerae* chromosomal DNA was purified according to Ausubel et al. (1990) from overnight cultures grown in LB at 37 °C. Standard methods were used for mini-preparations of plasmid DNA, restriction enzyme digestion, creation of blunt ends and ligations (Sambrook et al., 1989). Highly purified plasmid preparations were obtained by caesium chloride/ethidium bromide equilibrium centrifugation (Sambrook et al., 1989) or by using QIAEX columns (QIAGEN) in accordance with the manufacturer’s directions. DNA fragments were purified from agarose gels with GeneClean (Bio101) or with QIAEX beads (QIAGEN) in accordance with the supplier’s recommendations.

Preparation of *E. coli* competent cells and electroporation were carried out as described by Sambrook et al. (1989). *V. cholerae* competent cells were prepared and electroporated as described by Marcus et al. (1990). Briefly, cells were grown in LB to OD<sub>600</sub> 0·4–0·6 and centrifuged; the pellet was washed three times with 10% (v/v) glycerol containing 272 mM sucrose and finally resuspended in 1/100 of the original culture volume. Electroporations were carried out with a Bio-Rad Gene Pulsar with the Pulse controller set at 25 µF, 2·5 kV and 200 Ω. After electroporation, the suspension was diluted in 1 ml SOC (Sambrook et al., 1989), incubated in a shaker for 1 h at 37 °C and plated on appropriate selective media.

Southern blots were obtained by transferring DNA from agarose gels to nylon membranes (Hybond-N, Amersham) using 10 × SSC (Sambrook et al., 1989). Labelling of DNA probes with digoxigenin-11-dUTP (DIG) by random priming, hybridization and detection were carried out according to the labelling and detection kit instructions (Boehringer Mannheim). Prehybridization and hybridization were at 68 °C; membrane washings were under stringent conditions: two washes with 2 × SSC containing 0·1% SDS at room temperature and two washes with 0·1 × SSC containing 0·1% SDS at 68 °C.

PCR amplifications were performed in an automatic thermal cycler (Omnigene) using standard conditions (Innis & Gelfand, 1990). Oligonucleotide primers for PCR were as follows. For amplifications across the multiple cloning site of pUC19 and derivatives, P1L (5′-GGTTTTTCCGTCAC-GAGCTTGTG-3′) and P2L (5′-TATGTGTGTTGGAATTG-TGACGGG-3′) were used. For construction of the mutation in *phoB*<sup>Vc</sup>, JK3 (5′-GAAGATCTTGTGCCACTACCCACCA-3′) and JK4 (5′-GAAGATCTGAGCCGGAAGAT-GA-3′) were used. The oligonucleotides JK3 and JK4 span nt 1136–1119 and 1151–1168, respectively, in the 4·48 kb in-
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Source/reference</th>
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<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
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<tr>
<td>DH5α</td>
<td>F− endA1 hisdR17 (rE, mE) supE44 thi-1 recA1 gyrA (Nal') relA1 Δ(lacZYA-argF) U169 F80lacZΔM15</td>
<td>Raleigh et al. (1988)</td>
</tr>
<tr>
<td>SY327</td>
<td>F− Δ(lac pro) nalA argE (Am) rif araD recA56 ispir R6K</td>
<td>Miller &amp; Mekalanos (1988)</td>
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<tr>
<td>SM10</td>
<td>thi thr leu tonA lacY supE44::RP4-2Tc::Mu ispir R6K</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>V. cholerae strains</strong></td>
<td></td>
<td></td>
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<tr>
<td>569B</td>
<td>Classical Inaba</td>
<td>Nelson et al. (1976)</td>
</tr>
<tr>
<td>569BSR</td>
<td>Sm'</td>
<td>This work</td>
</tr>
<tr>
<td>CVD103</td>
<td>ΔctxA derivative of 569B</td>
<td>Levine et al. (1988)</td>
</tr>
<tr>
<td>CVD103SR</td>
<td>Sm', ΔctxA</td>
<td>This work</td>
</tr>
<tr>
<td>WK1</td>
<td>pboB mutant of CVD103; Sm', Km'</td>
<td>This work</td>
</tr>
<tr>
<td>WK2</td>
<td>pboB mutant of CVD103; Sm'</td>
<td>This work</td>
</tr>
<tr>
<td>WK3</td>
<td>pboB mutant of 569B; Sm', Km'</td>
<td>This work</td>
</tr>
<tr>
<td>WK4</td>
<td>pboB mutant of 569B; Sm'</td>
<td>This work</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pUC-4K</td>
<td>Km resistance gene of Tn903 in pUC4</td>
<td>Oka et al. (1981)</td>
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<tr>
<td>pJG9</td>
<td>Cm'</td>
<td>Galen &amp; Levine (1995)</td>
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<tr>
<td>pWK1</td>
<td>Km', Tc', low-copy cloning vector</td>
<td>Stoker et al. (1982)</td>
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<tr>
<td>pWK5</td>
<td>448 kb EcoRI insert of V. cholerae chromosome cloned into pUC19</td>
<td>This work</td>
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<tr>
<td>pWK5a</td>
<td>3.15 kb PstI fragment from pWK1 cloned into PstI site of PUC19</td>
<td>This work</td>
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<tr>
<td>pWK5b</td>
<td>2.2 kb BglII–EcoRI fragment from PCR amplification of pWK5 with P1L and JK4 cloned into BamHI/EcoRI sites of pUC19</td>
<td>This work</td>
</tr>
<tr>
<td>pWK5c</td>
<td>0.95 kb BglII–HindIII fragment from PCR amplification of pWK5 with P2L and JK3 cloned into BamHI/HindIII sites of pUC19</td>
<td>This work</td>
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<td>pWK6</td>
<td>2.2 kb XbaI–PstI fragment from pWK5a cloned into XbaI/PstI sites of pUC19</td>
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<td>pWK7</td>
<td>0.95 kb Smal–EcoRI fragment from pWK5b cloned into Smal/EcoRI sites of pWK5c</td>
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<td>pWK8</td>
<td>1.5 kb BamHI fragment from pUC-4K containing the Km' cassette cloned into the BamHI site of pWK6</td>
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<td>pWK9</td>
<td>4.65 kb EcoRI–EcoRV fragment from pWK7 blunt-ended and cloned into the EcoRV site of pGP704</td>
<td>This work</td>
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<td>pWK10</td>
<td>1.9 kb Smal–Xbol fragment from pJG9 containing the sacB gene blunt-ended and cloned into pWK9 cut with EcoRI and blunt-ended</td>
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<td>pWK11</td>
<td>2.7 kb EcoRI–EcoRV fragment from pWK6 cloned into pGP704 digested with EcoRI/EcoRV</td>
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The Vibrio cholerae PhoBR regulatory system

DNA was sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

The construction of clones for the complementation analysis, WK9 (5’-CGGAAATTCATCACCTCACCACAC-3’), which incorporates an EcoRI site extension, was used.

DNA was sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). To sequence the 4.48 kb fragment, the nested-deletion strategy with exonuclease III and S1 nuclease (Pharmacia) was used to generate a series of deletion clones that were then sequenced using the M13/pUC reverse and forward sequencing primers (Gibco-BRL). A primer-walking strategy was also used and primers, based on a reliable sequence obtained in a previous cycle, were designed to provide complete sequence data for both strands. Sequence reaction products were analysed with an ABI automated DNA sequencer (Protein and Nucleic Acids Chemistry Laboratory, Leicester University). Comparison of the nucleotides and derived polypeptides with sequences in the GenBank and EMBL databases were carried out by the use of FASTA (Pearson & Lipman, 1988) and BLAST (Altschul et al.,...
Cloning procedures. A 300 bp fragment containing a DNA sequence with homology to the phoB gene of *E. coli* was obtained by PCR amplification of chromosomal DNA of V. *cholerae* strain CVD103 (S. Humphreys & J. M. Ketley, unpublished results) using degenerate primers (PCR-DOP; Wren et al., 1992). The fragment was cloned into pUC19 to form pSH4. CVD103 chromosomal DNA was digested with several restriction enzymes and analysed by Southern hybridization using the 300 bp insert of pSH4 as a probe. The probe hybridized to single chromosomal fragments cut with EcoRI (448 kb) and BglII (65 kb). A library was constructed of size-selected (3.5–5.5 kb) CVD103 genomic DNA digested with EcoRI and cloned into the EcoRI site of pUC19. A clone was isolated that contained a plasmid with a 4.4 kb insert that hybridized with the probe. The plasmid was named pWK1.

For the complementation analysis, P2L and WK9 were used with pWK1 to amplify a fragment of about 1.75 kb that contained the phoB*野生* gene plus the regulatory region of the phoBR*野生* operon. Oligonucleotide WK9 spans nt 1723–1739 in the 4.4 kb insert of pWK1 and has an EcoRI site at its 5′ end. The amplified fragment was digested with EcoRI and cloned into pUC19 and pLG339, both digested with the same enzyme. Complementation tests were also done using pLG339 derivatives containing the 3.15 kb *PstI* fragment from pWK1 (nt 1–3150).

Mutant construction. The 3.15 kb *PstI* fragment from pWK1 was cloned into the *PstI* site of pUC19 to form pWK5. The primer pairs P1L/JK4 and P2L/JK3 were used with pWK5 to amplify by PCR two fragments of 2.2 kb and 0.95 kb, respectively. Each fragment contained part of the gene with a new BglII restriction site at one end and was subcloned via several steps into pUC19 (pWK5a–c; Table 1). The resulting plasmid, pWK6, contains the phoB*野生* gene with an internal deletion of 14 bp and a 26 bp insertion from pUC19 containing sites for the enzymes Smal, BglII and XbaI. Plasmid pWK6 was then digested with BglII and a 1.5 kb kanamycin-resistance cassette, derived from pUC4K, was cloned into the phoB*野生* gene to form pWK7.

A derivative of the suicide plasmid pGP704 was used to introduce the mutation into the chromosomal phoB*野生* gene. The 2.89 kb EcoRI–EcoRV fragment containing the mutated phoB*突变* gene from pWK7 was blunt-end ligated into the EcoRV site of pGP704, resulting in pWK8. To facilitate the selection of the mutant, the sacB gene of *Bacillus subtilis* was cloned into the construct. pJK9 was digested with Smal and XhoI and the 1.9 kb fragment containing the sacB gene was blunt-ended and ligated into the blunt-ended EcoRI site of pWK8 to form pWK9.

pWK9 was transformed into *E. coli* SY327 Δpir and then to SM10 Δpir for mobilization into streptomycin-resistant derivatives of *V. cholerae* strains CVD103 and 569B. Merodiploid transconjugants, the result of a single recombination event at the site of *V. cholerae* homologous sequences, were selected on medium containing ampicillin, streptomycin and kanamycin. Recombinants that had undergone a second recombination event were selected by growing the merodiploids firstly in LB with streptomycin and then in the same medium supplemented with 5% sucrose for several generations. The cells were plated on LA plates supplemented with streptomycin and sucrose and the colonies tested for ampicillin sensitivity and kanamycin resistance. Ampicillin-sensitive kanamycin-resistant cells were expected to possess the mutated copy of the putative phoB*突变* gene, whereas the ampicillin-sensitive kanamycin-sensitive cells were expected to be wild-type, generated by an alternative resolution of the merodiploids. Kanamycin-resistant insertion mutants in the phoB*突变* gene constructed in strains CVD103SR and 569BSR were named WK1 and WK3, respectively. The formation of the merodiploid, the insertion of the kanamycin cassette in the phoB*突变* gene and the recovery of the wild-type genotype were confirmed by Southern hybridization in which the 3.15 kb *PstI* fragment from pWK5 was used as the probe.

Mutants not containing the kanamycin-resistance cassette in the *V. cholerae* phoB*野生* ORF were also constructed. Firstly, the 2.89 kb EcoRI–EcoRV fragment from pWK6 containing the mutated phoB*突变* gene was cloned into pGP704 digested with the same enzymes, forming pWK10. The sacB gene on the 1.9 kb BamHI–EcoRV fragment from pJK9 was then cloned into pWK10 digested with EcoRV and BglII, resulting in pWK11. This plasmid was used to construct new mutants in the *V. cholerae* strains CVD103SR and 569BSR as described above for WK1 and WK3. The BamHI site introduced during the mutation of the phoB*突变* gene was used to differentiate between the mutant and wild-type genotypes. The formation of the merodiploids and of the putative phoB*突变* mutants, and the recovery of the wild-type genotype, were confirmed by Southern hybridization. The kanamycin-sensitive phoB*突变* mutants in CVD103SR and 569BSR were named WK2 and WK4, respectively.

Alkaline phosphatase assay. Levels of alkaline phosphatase activity were determined by a variation of the permeabilized whole-cell assay (Gutiérrez et al., 1987). The optical density of the culture at 600 nm was measured and a determined volume of the culture was centrifuged. The pellet was resuspended in 0.1 M Tris/HCl, pH 8.0, and the cells were permeabilized by addition of hexadecyltrimethylammonium bromide (CTAB; final concentration, 0.025%) followed by 10 s vortexing. The reaction was carried out at 37 °C by the addition of ONPG (Sigma; final concentration, 0.04%) and stopped with K2HPO4 at 0.1M. The units of alkaline phosphatase were calculated using the formula 10×[Afi/Ami–(1.75 × A550)]/t × OD0.045 × V, where Afi and Ami are the absorbancies of the reaction mix after an incubation time t (min), OD0.045 is the optical density of the culture and V is the volume (ml) of the culture used in the assay. The activity measured was expressed in Miller units (Miller, 1972).

Protein analysis. Proteins from whole-cell lysates were analysed by electrohoresis through 11% polyacrylamide (T = 10%, C = 16%) SDS gels as previously described (Laemmli, 1970). Outer-membrane proteins prepared by differential solubilization of cell envelopes with Triton X-100 (Snaitman, 1974) were analysed by SDS-PAGE with 10% running gels. Periplasmic proteins were obtained by centrifugation of a suspension of spheroplasts prepared as described by Roy et al. (1982a). The resulting supernatant was precipitated and the periplasmic proteins were analysed by SDS-PAGE with 11% running gels as above. Gels were stained with Coomassie brilliant blue R 250 (Bio-Rad). Protein concentration was estimated by the method of Bradford (1976).

Two-dimensional polyacrylamide gel electrophoresis was performed with a Multiphor II horizontal unit (Pharmacia) on an immobilized pH gradient 3–10 (Immobiline DryStrip pH3–10L) for the first dimension and SDS-PAGE on a 12–14% gradient gel (ExcelGel XL) for the second dimension. Samples were prepared as recommended by the supplier by...
resuspending cells in lysis buffer (8.99 M urea, 0.02% Triton X-100, 0.13 M DTT, 0.02%, v/v, Pharmalyte 3-10, 8 mM PMSF) for 2 h at room temperature, followed by ultracentrifugation (250 000 g). A volume containing approximately 20 µg proteins in sample buffer was loaded onto the strip and the proteins were focussed at 1 mA for 20 h at 20 °C. The gel strip was then loaded onto the polyacrylamide-SDS slab gel and electrophoresed at 600 V for 3.5 h. Gels were then fixed and silver stained.

**CT ELISA assay.** *V. cholerae* strains were grown in TGH and TGLP, pH 6.5, at 30 °C with aeration for approximately 18 h (DiRita et al., 1990). The cells were centrifuged and the toxin in the supernatant was assayed by GM1-ELISA (Holmgren, 1973).

**Conjugation.** *V. cholerae* strains CVD103SR and 569BSR were mated with *E. coli* SM10/spir harbouring either pWK9 or pWK11 by mixing an equal number of exponential-phase cells. The mating mixture was spotted onto a LA plate and incubated overnight at 37 °C. Dilutions of the mixture were plated on LA containing ampicillin, kanamycin, and streptomycin or ampicillin and streptomycin for the selection of the transconjugants derived from pWK9 or pWK11, respectively. Transconjugants were confirmed as *V. cholerae* by a positive Kovac oxidase reaction (Kay et al., 1994).

**Rabbit ileal loop anastomosis test (RILAT).** A competitive colonization assay (Ketley et al., 1993) was carried out using RILAT (Ketley et al., 1987) with the following modifications. The peritoneal cavity was opened and the washed intestine was clamped with intestinal clamps and resected 10 cm proximal to the ileo-caecal junction. Moving proximal to the resection, the length of intestine required for the construction of ligated loops was measured and the intestine clamped and resected; the isolated segment of intestine was temporarily resected; the isolated segment of intestine was temporarily blocked and ligated sample loops and spacer loops constructed and inoculated as before. After closing the laparotomy, the rabbit was allowed to recover before termination of the procedure 18 h after loop inoculation.

The samples inoculated into ligated loops were prepared as follows. *V. cholerae* strains were grown to mid-exponential phase in LB at 37 °C, the cultures were then centrifuged and the pellets resuspended in saline. The OD_{600} of the suspensions was obtained and they were diluted (based on previously determined growth curves) to give the required number of cells to be inoculated in 500 µl saline. Cells were also plated for viable counts. The two test strains were inoculated separately or equal numbers of cells were combined to the same total count and inoculated to assess the ability to colonize competitively. Three inoculum sizes were used: 2 × 10^{8}, 2 × 10^{7} and 2 × 10^{6} cells in 500 µl saline. In some experiments, the cells were resuspended in saline supplemented with KH_{2}PO_{4}, at a final concentration of 6.5 mM. The samples were injected into 5 cm intestinal loops. Saline was used as a negative control and a loop containing CT (0.5 µg) was included to check the physiological response of the tissue. On termination of the procedure, a small piece of tissue from each loop was removed, weighed, washed three times with saline, transferred to 2 ml saline and homogenized. Appropriate dilutions were plated onto LA and LA containing streptomycin to determine the total number of vibrios and to allow differentiation of the strains. The ratios of the antibiotic-sensitive (parental strain) to -resistant colonies (mutants) per gram of tissue were determined.

**RESULTS**

**Cloning and sequence analysis of the phoB<sup>¾</sup> and phoR<sup>¾</sup> genes**

A 300 bp fragment containing a DNA sequence with homology to the phoB gene of *E. coli* (S. Humphreys & J. M. Ketley, unpublished results) was used to probe a size-selected (3.5–5.5 kb) library of strain CVD103 genomic DNA in pUC19. A plasmid (pWK1) that contained a 4.48 kb insert was isolated.

Nucleotide sequence analysis of the insert in pWK1 (accession number AF043352) revealed the presence of four ORFs. One of them, 0.69 kb long, contained the same nucleotide sequence as the 300 bp fragment used as a probe. The deduced amino acid sequence resulted in a 229 residue protein that is the same length and bears approximately 72% identity with PhoB of *E. coli* (Makino et al., 1986a). The *V. cholerae* PhoB sequence (here designated as PhoB<sup>vc</sup>) contains conserved amino acid residues and two blocks of four hydrophobic residues found in all the OmpR-like subfamily sequences (Stock et al., 1989). The corresponding gene, phoB<sup>vc</sup>, is preceded by a Shine–Dalgarno sequence (AGG; Shine & Dalgarno, 1975), a Pribnow box and a short sequence with extensive sequence identity to the phosphate box (Pho box; Makino et al., 1986a) in the regulatory region of the *E. coli* phoB gene (Fig. 1a).

A second ORF (phoR<sup>vc</sup>), 1.3 kb long, was found with an ATG 21 bp downstream from the translation stop codon of phoB<sup>vc</sup>. The deduced amino acid sequence is a
protein of 430 residues of the same length and 58.1% identical to the phosphate-regulon HK sensor, PhoR, from *E. coli* (Makino *et al.*, 1986b). In addition to the putative site of auto-phosphorylation at His-215, the *V. cholerae* PhoR (here designated as PhoR\(^{vc}\)) contains sequences that are highly conserved within the C-terminal region of the HK family. PhoR of *E. coli* has an extended hydrophobic region (residues 10–60) that is likely to be membrane associated (Makino *et al.*, 1986b). A hydrophobic map of PhoR\(^{vc}\) (PROWL; Rockefeller University, USA) revealed the presence of a similar sized hydrophobic sequence in the same amino-terminal region of the protein (results not shown); therefore, PhoR\(^{vc}\) is also probably a membrane-associated protein.

**Mutation of the phoB\(^{vc}\) gene**

A unique BamHI site engineered into the phoB\(^{vc}\) gene (pWK6; Fig. 1b) was used to insert a kanamycin-resistance cassette (pWK7). The mutated fragment was then cloned into the suicide vector pGP704 containing a sacB gene (pWK9). After introduction of pWK9 into *V. cholerae*, a phoB\(^{vc}\) merodiploid was isolated by selection for kanamycin resistance and the loss of the sacB gene, and used to obtain a mutant containing the resistance cassette in phoB\(^{vc}\). Kanamycin-resistant mutants were constructed in *V. cholerae* strains CVD103SR and 569BSR, and designated WK1 and WK3, respectively. The insertion of the kanamycin-resistance cassette into phoB\(^{vc}\), and absence of vector sequences in WK2 and WK4 was confirmed by Southern hybridization (data not shown).

In-frame deletion mutants in phoB\(^{vc}\) that did not contain the kanamycin-resistance cassette were constructed in strains CVD103SR and 569BSR and named WK2 and WK4, respectively. The phoB\(^{vc}\) mutated fragment (Fig. 1b) from pWK6 was subcloned directly into pGP704 containing sacB (pWK11). Following the introduction of pWK11 into *V. cholerae*, phoB\(^{vc}\) merodiploids were isolated and used to obtain new phoB\(^{vc}\) mutants by selection for the loss of the sacB gene. The insertion of the BamHI site associated with the in-frame mutation and absence of vector sequences in WK2 and WK4 was confirmed by Southern hybridization (data not shown).

**Growth and properties of the phoB\(^{vc}\) mutants**

A mutation in the phoB\(^{vc}\) gene would be expected to have physiological consequences with respect to phosphate metabolism. Accordingly, growth ability at 37 °C was examined in liquid cultures in LB and in TGHP or TGLP media at pH 8.0 (Fig. 2). No significant reduction in growth rates or final cell-culture densities was seen for the four mutants in comparison to the parent strains when they were grown in LB (Fig. 2a and data not shown) or in TGHP (Fig. 2b and data not shown). The growth rate in LB was greater than that in TGHP and the cultures reached higher cell densities at stationary phase. In contrast, when the mutant and parent strains were grown under phosphate-limited conditions (TGLP) all strains showed a reduction in both growth rate and cell density at stationary growth phase in comparison to growth in LB or TGHP. More importantly, in TGLP a marked difference was observed in the growth rate and cell density of mutants WK2 and WK4 (Fig. 2b) and WK1 and WK3 (data not shown) when compared to the parental strains. No difference was observed between the phoB\(^{vc}\) mutants in the same *V. cholerae* strain constructed by different strategies.

**Expression of members of the Pho\(^{vc}\) regulon**

In *E. coli*, limitation of inorganic phosphate results in the synthesis of a number of proteins important for acquiring phosphate (Wanner, 1996). One such member of the *E. coli* Pho regulon is alkaline phosphatase (PhoA). When grown under phosphate-limiting conditions, *V. cholerae* produces a 60 kDa periplasmic polypeptide with alkaline phosphatase activity (Roy *et al.*, 1982a). Synthesis of this enzyme by mutant and parent strains of *V. cholerae* was examined in periplasmic cell fractions of strains grown in TGHP or
The *Vibrio cholerae* PhoBR regulatory system

**Table 2.** Effect of inorganic phosphate in the growth medium on alkaline phosphatase and cholera toxin expression in *phoB*-like mutants and the parent strain

All strains were grown at 30 °C. TGHP (high phosphate) and TGLP (low phosphate) at pH 6.5, supplemented with amino acids, were used as growth media.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>PhoA (Miller units)*</th>
<th>CT (ng/OD&lt;sub&gt;600&lt;/sub&gt;)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>569BSR</td>
<td>TGHP</td>
<td>2.7 ± 1.2</td>
<td>241 ± 40</td>
</tr>
<tr>
<td>WK3</td>
<td>TGHP</td>
<td>2.5 ± 1.4</td>
<td>242 ± 20</td>
</tr>
<tr>
<td>WK4</td>
<td>TGHP</td>
<td>3.1 ± 1.5</td>
<td>264 ± 35</td>
</tr>
<tr>
<td>569BSR</td>
<td>TGLP</td>
<td>82.9 ± 7.6</td>
<td>92 ± 18</td>
</tr>
<tr>
<td>WK3</td>
<td>TGLP</td>
<td>21 ± 0.5</td>
<td>93 ± 14</td>
</tr>
<tr>
<td>WK4</td>
<td>TGLP</td>
<td>2.9 ± 0.6</td>
<td>81 ± 14</td>
</tr>
</tbody>
</table>

* Miller units are proportional to the increase in o-nitrophenol min<sup>−1</sup> (OD unit)<sup>−1</sup>. Values represent mean ± standard deviation for five independent experiments.

‡ Amount of toxin produced (ng) divided by optical density of the culture, calculated from a CT standard curve and assayed by GM1-ELISA. Values represent mean ± standard deviation for four independent experiments.

TGLP pH 8.0, at 37 °C (Table 2). PhoA activities were only detected in the periplasm of the parent strains grown in TGLP; no induction of PhoA synthesis was observed in WK3 and WK4 grown under low- or high-phosphate conditions. These data show that in *V. cholerae*, a functional PhoB<sub>vc</sub> protein is also essential for the expression of alkaline phosphatase activity.

In addition to PhoA, under conditions of limited inorganic phosphate, *E. coli* synthesizes an outer-membrane protein, PhoE, and components of high-affinity transport systems for inorganic phosphate and sn-glycerol 3-phosphate (Wanner, 1996). To check whether the protein profiles produced by *phoB<sup>vc</sup>* mutants were influenced by the concentration of inorganic phosphate in the growth medium, *V. cholerae* was grown in TGHP and TGLP pH 8.0. The protein profiles of whole cells, outer-membrane fractions and periplasmic fractions were examined by SDS-PAGE. Fig. 3(a) shows the electrophoretic pattern of whole-cell lysates from strain 569BSR and mutants WK3 and WK4. No major differences can be seen among the protein profiles for the cells grown in TGHP. Under limiting-phosphate conditions, in comparison to 569BSR, at least four proteins, with molecular masses of 60 kDa, 51 kDa, 38 kDa and 31 kDa, are either absent or reduced in the lysates of the mutants grown under the same conditions. Comparison of outer-membrane protein profiles of cells grown in TGHP and TGLP revealed that the protein of 38 kDa synthesized by the strain 569BSR is an outer-membrane protein (Fig. 3b). In addition, a protein of 41 kDa appears to be expressed at higher levels in the mutants. Proteins of 60 kDa, 31 kDa and 51 kDa, produced under the same conditions, appear to be periplasmic (Fig. 3c).

A more accurate analysis of differences in protein expression between 569BSR and the *phoB<sup>vc</sup>* mutants WK3 and WK4 grown under phosphate-limited conditions was carried out by two-dimensional gel electro-
Fig. 4. Two-dimensional gel patterns of proteins synthesized by strain 569BSR (a), and phoB mutants WK3 (b) and WK4 (c) under limited-phosphate conditions (TGLP, pH 8.0). Total cell proteins were separated by two-dimensional gel electrophoresis with a pH gradient of 3–10 (right to left) and visualized by silver staining. Major proteins were numbered arbitrarily; their molecular masses and pIs are detailed in Table 3. Molecular mass markers (kDa) are indicated on the left and pl values estimated from the IEF strip are indicated at the top.

Table 3. Differences in protein expression between wild-type and phoB<sup>vc</sup> mutant V. cholerae in low-phosphate medium

<table>
<thead>
<tr>
<th>Protein no.</th>
<th>kDa</th>
<th>pl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27-2</td>
<td>7.7</td>
</tr>
<tr>
<td>2</td>
<td>27-5</td>
<td>7.4</td>
</tr>
<tr>
<td>3</td>
<td>42.8</td>
<td>7.5</td>
</tr>
<tr>
<td>4</td>
<td>41.6</td>
<td>6.8</td>
</tr>
<tr>
<td>5</td>
<td>60.5</td>
<td>7.1</td>
</tr>
<tr>
<td>6</td>
<td>57.5</td>
<td>7.1</td>
</tr>
<tr>
<td>7</td>
<td>35.5</td>
<td>6.5</td>
</tr>
<tr>
<td>8</td>
<td>61.1</td>
<td>6.4</td>
</tr>
<tr>
<td>9</td>
<td>61.1</td>
<td>6.5</td>
</tr>
<tr>
<td>10</td>
<td>66.1</td>
<td>6.2</td>
</tr>
<tr>
<td>11</td>
<td>53.2</td>
<td>6.4</td>
</tr>
<tr>
<td>12</td>
<td>38.8</td>
<td>5</td>
</tr>
</tbody>
</table>

Listed proteins were consistently absent or poorly expressed in phoB<sup>vc</sup> strains in comparison to wild-type V. cholerae. The data shown were obtained from Fig. 4.

Evaluation of the role of the phoB<sup>vc</sup> gene in CT expression

The production of CT by 569BSR and the phoB<sup>vc</sup> gene mutants WK3 and WK4 was examined by GM1-ELISA (Holmgren, 1973). The assay was carried out on the supernatant of cultures grown at 30 °C in TGHP and TGLP at pH 6.5 in the presence or absence of a mixture of amino acids. The results are summarized in Table 2. As expected from published work (Sagar <i>et al.</i>, 1981), in the absence of amino acids the cells grew very poorly in both media and produced no toxin (data not shown). In the presence of amino acids and at the high-phosphate concentration, the strains produced three- to fourfold more toxin than under limited-phosphate conditions. No significant difference was seen between the ability of the parental and mutant strains to produce CT under low- or high-phosphate conditions.
colonizes more efficiently than the resistant strain. A ratio bigger than 1 indicates that the sensitive strain colonizes more efficiently than the mutant when the cells were inoculated in saline.WK2, CVD103 colonized about 10-fold better than the mutant. As expected, similarities were found with phoBR of E. coli. The phoBR regulatory region contains a putative consensus Pho box located at region—35 of the promoter, suggesting that phoBR is under control of PhoB and therefore, similar to E. coli, is phosphate regulated. The two ORFs, phoB and phoR, found downstream of the regulatory region are organized in the same way as in E. coli. There seems to be no consensus promoter sequence in the intergenic region and a putative stem-loop structure that could function as a transcription terminator was found downstream of the phoR coding region. Therefore, it seems likely that the two ORFs are co-transcribed and that the V. cholerae pho operon consists of two cistrons, phoB and phoR. The stem-loop structure identified within phoR may function as a transcriptional attenuator. Both the presence of this possible structure and the poor consensus Shine-Dalgarno sequence that precedes the phoR gene suggest that phoR is expressed at a lower level than phoB. This bears similarity with both E. coli phoBR (Makino et al., 1986b; Haldimann et al., 1998) and ompR-envZ (Comeau et al., 1985) operons, where expression of the HK gene (phoR or envZ) is lower than the cognate RR gene. At the deduced amino acid sequence level, both PhoB and PhoR bear extensive homologies with their counterparts from E. coli.

In addition to phoB and phoR, two incomplete ORFs, orf1 and orf4 were identified in the 4.48 kb chromosomal DNA fragment in pWK1. One was found in the EcoRI–DreI fragment (nt 1–870); it starts with an ATG 236 nt upstream of the phoB translation start codon and would be translated in the opposite direction to phoBR. The incomplete orf1 would encode a polypeptide 238 residues long which is 57% identical to a hypothetical protein from the EMBL/GenBank data-

### Table 4. Colonization efficiency of V. cholerae strains CVD103, 569B, WK2 and WK4

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Buffer*</th>
<th>Ratio†</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVD103/CVD103SR</td>
<td>Saline</td>
<td>1:5</td>
</tr>
<tr>
<td>CVD103/WK2</td>
<td>Saline</td>
<td>10:5</td>
</tr>
<tr>
<td>CVD103/WK2</td>
<td>Saline/HP</td>
<td>4:3</td>
</tr>
<tr>
<td>569B/WK4</td>
<td>Saline</td>
<td>27</td>
</tr>
<tr>
<td>569B/WK4</td>
<td>Saline/HP</td>
<td>12</td>
</tr>
</tbody>
</table>

* Buffer used to resuspend the cells to be inoculated into the rabbit; HP is KH₂PO₄ 6.5 mM.
† Ratio of streptomycin-sensitive to streptomycin-resistant organisms. A ratio bigger than 1 means that the sensitive strain colonizes more efficiently than the resistant strain.

**PhoB** has a role in intestinal colonization

Colonization of the small intestine by V. cholerae strains CVD103 and 569B and their mutants, WK2 and WK4 respectively, was studied in the adult rabbit using ligated ileal loops. The results are summarized in Table 4. Comparison of CVD103 and its streptomycin-resistant derivative (the parental strain of WK2) was carried out by inoculating an equal number of c.f.u. in intestinal loops of the rabbit. After 18 h, the number of c.f.u. associated with intestinal tissue and the ratio of the two inoculated strains were determined. As previously observed (Ketley et al., 1993), strain CVD103SR colonizes the small intestine of the rabbit slightly less efficiently than the parent strain CVD103. In a competitive assay between CVD103 and the phoB mutant WK2, CVD103 colonized about 10-fold better than the mutant when the cells were inoculated in saline. However, when the cells were in saline supplemented with phosphate (6.5 mM KH₂PO₄), the colonizing ability of the mutant in comparison to CVD103 improved markedly. In a similar competitive assay, the colonization abilities of the strain 569B and of the phoB deletion mutant WK4 were compared. When inoculated in saline, strain 569B colonized the small intestine of the rabbit 27-fold better than the mutant. As with the CVD103/WK2 comparison, in the presence of high phosphate levels, the colonization ratio dropped. These results indicate that the mutation of the phoB gene affects the colonization ability of V. cholerae. This reduction in colonization ability can be partly reversed by increasing the luminal concentration of inorganic phosphate.

**Complementation studies**

To determine if the wild-type phenotype could be restored by reintroducing functional phoB gene, the mutants were transformed with the 3.15 kb PstI fragment encoding phoBR operon cloned into the low-copy-number vector pLG339. The transformants were able to produce the same level of PhoA and present the same protein pattern of the parent strain under low-phosphate growth conditions (data not shown). Attempts were also made to complement the mutants with a fragment containing the whole phoB gene plus sequences upstream of the start codon (948 nt) and downstream of the stop codon (30 nt) cloned in pUC19 and also in pLG339. In neither case, however, was the wild-type phenotype restored.

**DISCUSSION**

In E. coli, alkaline phosphatase (PhoA) has an important role in phosphate metabolism, supplying cells with inorganic phosphate from exogenous sources (Torriani, 1960). The phoA gene is a member of the Pho regulon, as are several other genes whose products have roles in inorganic phosphate assimilation (Wanner, 1996). It is known that V. cholerae, like E. coli, synthesizes PhoA when grown under phosphate-limiting conditions (Roy et al., 1982a, b); however, none of the genes of the Pho regulon in V. cholerae have been characterized.

In this work, we report the cloning and sequencing of the phoBR operon of V. cholerae. As might be expected, similarities were found with phoBR of E. coli. The phoBR regulatory region contains a putative consensus Pho box located at region—35 of the promoter, suggesting that phoBR is under control of PhoB and therefore, similar to E. coli, is phosphate regulated. The two ORFs, phoB and phoR, found downstream of the regulatory region are organized in the same way as in E. coli. There seems to be no consensus promoter sequence in the intergenic region and a putative stem-loop structure that could function as a transcription terminator was found downstream of the phoR coding region. Therefore, it seems likely that the two ORFs are co-transcribed and that the V. cholerae pho operon consists of two cistrons, phoB and phoR. The stem-loop structure identified within phoR may function as a transcriptional attenuator. Both the presence of this possible structure and the poor consensus Shine-Dalgarno sequence that precedes the phoR gene suggest that phoR is expressed at a lower level than phoB. This bears similarity with both E. coli phoBR (Makino et al., 1986b; Haldimann et al., 1998) and ompR-envZ (Comeau et al., 1985) operons, where expression of the HK gene (phoR or envZ) is lower than the cognate RR gene. At the deduced amino acid sequence level, both PhoB and PhoR bear extensive homologies with their counterparts from E. coli.

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bases of 303 residues that maps in the intergenic region araI–aroM of E. coli. The other ORF, orf4, is incomplete and is in the 1.2 kb PstI–EcoRI fragment (nt 3150–4480) of pWK1, downstream of the pboR<sup>vc</sup> gene. This ORF possesses a high level of similarity to the E. coli ppx gene, which is thought to be a member of the Pho regulon (Kornberg, 1994). Ppx is an exopolyphosphatase that acts on the ends of polyphosphate chains, progressively removing orthophosphate residues and, thus, provides an alternative source of organic phosphate to ATP.

In E. coli, mutations in pboB can inhibit the transcription of the Pho regulon genes (Bracha & Yagil, 1973; Morris et al., 1974; Yagil et al., 1975). Defined mutation of pboB<sup>vc</sup> generated strains that differed markedly from their parents. Under phosphate-limiting conditions they grew very poorly and did not express alkaline phosphatase, an enzyme synthesized in high levels by the parental strains under the same growth conditions. The poor growth of the pboB<sup>vc</sup> mutants under phosphate limitation correlates with the inability to express alkaline phosphatase and other proteins synthesized by the parental strains that may be involved in the phosphate metabolism. The pattern of protein expression by the pboB<sup>vc</sup> mutants in limiting-phosphate conditions was also different to that obtained with the parent strain. The 60 kDa protein is likely to be PhoA, as it is periplasmic and has the same apparent molecular mass as the PhoA protein described previously in V. cholerae (Roy et al., 1982a). The 31 kDa and 51 kDa proteins, by analogy with periplasmic proteins from E. coli (Tommassen & Lugtenberg, 1980) induced under the same conditions, are probably involved in binding and transport of phosphorus compounds. The outer-membrane protein of 38 kDa is likely to be the homologue of the E. coli porin PhoE (VanBogelen et al., 1996). The V. cholerae homologue is approximately the same molecular mass and is associated with peptidoglycan (data not shown), a property of proteins that form channels in the outer membrane (Nikaido, 1983). A major outer-membrane protein of 33-9 kDa with similar characteristics was identified in Vibrio parahaemolyticus also grown under limiting-phosphate conditions (Mccarter & Silverman, 1987). As no function has been assigned to the majority of proteins differentially expressed in the parental strain under low-phosphate conditions, further work is necessary in order to characterize the V. cholerae Pho regulon.

Both sets of pboB<sup>vc</sup> mutants could be complemented in trans by the entire pboBR<sup>vc</sup> operon cloned into a low-copy-number plasmid. The fact that the wild-type phenotype was not restored by in trans complementation with just the pboB<sup>vc</sup> gene with regulatory region is intriguing. With respect to the mutants WK1 and WK3, one can argue that the insertion of the kanamycin cassette resulted in a polar effect on pboR<sup>vc</sup> gene expression. In contrast, mutants WK2 and WK4 have small in-frame alterations in the pboB<sup>vc</sup> DNA sequences, and one should not expect that such mutations would cause a polar effect on pboR<sup>vc</sup>. The lack of complementation by the wild-type pboB<sup>vc</sup> gene plus regulatory region suggests that the in-frame mutation in WK2 and WK4 is dominant.

The biosynthesis of CT is not affected by the pboB<sup>vc</sup> mutation, an indication that its expression is not also under the control of PhoBR<sup>vc</sup>. It has been known for some time that environmental signals such as temperature, pH, osmolarity and amino acids, among others, cause changes in the expression of CT (Miller & Mekalanos, 1988). The diminished expression of CT by the pboB<sup>vc</sup> mutants and the parent strain in TGLP/ amino acids medium was expected. The importance of phosphate in the stimulation of amino acid uptake in minimal medium and, consequently, in growth and expression of CT by V. cholerae has been reported previously (Sagar et al., 1981).

The mutation of the pboB<sup>vc</sup> gene affects intestinal colonization of adult rabbit intestine by V. cholerae. The effect was greater on pboB<sup>vc</sup> mutants derived from the toxigenic strain 569B, probably because CVD103, a ctxA derivative, is less able to colonize the intestine (Levine et al., 1988). It is not known which members of the Pho regulon of V. cholerae are involved in intestinal colonization, or how. In P. aeruginosa the phosphate-regulated haemolytic phospholipase C has been shown to be involved in colonization (Ostroff et al., 1989). Vibrios also produce phospholipases, some of which are induced in phosphate-deficient conditions; however, no involvement of such proteins with pathogenesis has been reported (McCarter & Silverman, 1987; Fiore et al., 1997).

The addition of phosphate at a high concentration to the inoculation medium recovered partially the ability of the mutants to colonize the rabbit small intestine. Mutants and parent strain do not grow at the same rate in low phosphate in vitro but there is no difference in their growth rate in high-phosphate conditions (Fig. 2b). Therefore, one could expect that under phosphate-replete conditions, the mutant and parent strain would colonize the rabbit intestinal mucosa with equal ability. The decreased colonization of the mutant in high-phosphate conditions suggests that member(s) of the Pho<sup>vc</sup> regulon may have a role in intestinal colonization. Thus, the control of expression of pboBR<sup>vc</sup>, or indeed other members of the Pho regulon, in vivo may not be only in response to phosphate levels. It is known that a number of stimuli other than phosphate can induce components of the Pho regulon in a phosphate-dependent and -independent manner (Wanner & McSharry, 1982; VanBogelen et al., 1996). Accordingly, in E. coli the expression of several phosphate-regulated promoters can differ depending on their specific molecular controls. A pboB mutation in E. coli K-12 nearly abolishes phoA transcription, but the induction of another phosphate-regulated gene, psiE, is only reduced about tenfold (Wanner, 1986). Similarly, the mutation in a Pho regulon gene enhances enteroinvasive E. coli invasion, suggesting that Pho regulon genes are expressed in vivo (Sinai & Bavoil, 1993). Furthermore, transcription of himA, a phosphate-starvation-induced
gene (Wanner, 1986), has been shown to be selectively expressed by Salmonella cells grown in vivo (Mahan et al., 1993).

Intestinal colonization is a multistep process involving a hierarchy of events in which the vibrios in the gut overcome the mucus barrier, adhere to the mucosa, increase in number and detach (Benitez, 1997). The V. cholerae PhoBR TCR system may play a role in this complex process by regulating the expression of members of the Pho regulon that are involved in adaptive responses required for intestinal colonization. Given the important role of ToxR in V. cholerae pathogenesis, it is possible that there is some overlap between these two regulatory systems.

ACKNOWLEDGEMENTS

We are grateful to CAPES (Brazil) for a Fellowship to W.M.A.v.K., the Medical Research Council (UK) for a Studentship for S.H. and The Royal Society for a University Research Fellowship to J.M.K. We thank Professor J. Kaper for V. cholerae strains and Dr J. Galen for pG9. We gratefully acknowledge the technical assistance of Biomedical Services (University of Leicester) staff with the in vivo experiments.

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The *Vibrio cholerae* PhoBR regulatory system


Received 28 January 1999; revised 16 April 1999; accepted 21 April 1999.