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Phosphate Regulation of Gene Expression in *Vibrio parahaemolyticus*

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The synthesis of a major outer membrane protein, OmpP, in *Vibrio parahaemolyticus* was induced by growth in media deficient in phosphate. The gene, *ompP*, encoding this protein was cloned. Synthesis of OmpP in *Escherichia coli* was regulated by the availability of phosphate, and this control required the function of *pho* regulatory genes of *E. coli*. Analysis of gene fusion strains constructed by mutagenesis with transposon mini-Mu*lux* revealed that *ompP* was transcriptionally regulated in *V. parahaemolyticus*. Impaired growth of a strain with an *ompP* defect was observed in media which contained large linear polyphosphates as the phosphate source. This and other evidence suggested that OmpP functions as a porin channel for the entry of phosphate into the cell. A number of other proteins or activities were induced by phosphate limitation including hemolysin, phospholipase C, and phosphatase activities. A regulatory locus controlling expression of phosphate-regulated genes was identified and cloned. This regulatory locus cloned from *V. parahaemolyticus* was shown to complement *E. coli* strains with defects in *pho* regulatory genes.

Vibrio parahaemolyticus can be found in a variety of marine and estuarine habitats: free swimming in coastal waters, in association with zooplankton and other marine organisms, or attached to a variety of animate and inanimate objects (7, 24, 25). *V. parahaemolyticus* can also be a pathogen: it is a major cause of food poisoning in certain parts of the world (7, 43). To survive and propagate in different and changing environments, *Vibrio* species and other bacteria have the capacity to change their metabolic processes and structural characteristics in response to external cues. At the gene level, interacting global control networks operate to balance the biochemical economy of the cell. These allow differing sets of genes to be expressed in response to differing demands on the cell. One such regulatory system is the phosphate regulon. Limitation of inorganic phosphate results in the synthesis of a battery of proteins important for acquiring phosphate. In *Escherichia coli* these include alkaline phosphatase, components of a high-affinity inorganic phosphate transport system, *sn*-glycerol-3-phosphate transport proteins, and an outer membrane protein (20, 40). Wanner and McSharry (42) have identified at least 18 phosphate starvation-inducible (*psi*) loci using Mu d(*lac*) fusion technology. In *Pseudomonas aeruginosa* phosphate-regulated gene products include an outer membrane protein, alkaline phosphatase, and two hemolysins (22, 23). One of the hemolysins has phospholipase C activity (4), and the other is a a heat-stable glycolipid (37). Heat-stable hemolysin is thought to aid solubilization of phospholipids. Phospholipase C hydrolyzes phospholipids to liberate phosphorylcholine, which is a suitable substrate for the phosphatase. Thus, these gene products may function cooperatively to provide phosphate (27).

In this report, we show that induction of the phosphate regulon of *V. parahaemolyticus* leads to production of a major outer membrane protein (OmpP), hemolysin, phosphatase, and phospholipase C activities. Particular emphasis was given to a genetic analysis of *ompP* which included cloning the structural gene and construction of gene fusions. A regulatory locus required for expression of phosphate-controlled genes was also isolated and characterized. Phosphate-regulated proteins working together to scavenge phosphate could lead to an increased capacity for virulence as well as enhanced survival of the bacterium in the marine environment.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and cloning vectors. Strains, plasmids, and cloning vectors are listed in Table 1. The *V. parahaemolyticus* strains constructed for this work were derived from LM1, a spontaneous rifampin-resistant derivative of wild-type strain BB22. The cosmid bank was maintained in *E. coli* YMC10 *recA*.

Media. *V. parahaemolyticus* strains were routinely grown at 30°C in heart infusion medium (25 g of heart infusion broth [Difco Laboratories, Detroit, Mich.], 15 g of NaCl per liter). Cultures were also grown in 2216 medium (28 g of marine broth 2216 [Difco] per liter). Solid marine medium was supplemented with 20 g of agar per liter. Marine tetracycline-sensitive medium was made according to the formula of Bochner et al. (9) with the following modifications: 20 g of agar, 10 g of tryptone, 20 g of NaCl, and 2 ml of fusaric acid (2 mg/ml) per liter. For low-phosphate culture conditions the MOPS (morpholinepropanesulfonic acid) medium of Neidhardt et al. (33) was used. A 10-fold concentrate was prepared by the method of Bochner and Ames (8) with complete omission of phosphate. Minimal marine MOPS medium also contained 0.3% glycerol, 5 μg of thiamine per ml, and 0.34 M NaCl. Complete marine MOPS medium was minimal marine MOPS medium supplemented with 0.15% Casamino Acids (Difco) and 25 mM $MgSO_4$ (the approximate phosphate concentration provided by the Casamino Acids was 0.5 mM). Phosphate (K_2HPO_4) was added to media as indicated. LB (31) was used for maintenance of *E. coli* strains. Defibrinated sheep erythrocytes (Colorado Serum Co.) were washed two times with 0.85% saline and used in plates at a concentration of 10% (equivalent to 5% packed cells). Sodium polyphosphates were obtained from Sigma Chemical Co. (St. Louis, Mo.). Antibiotics (Sigma) were added to the media at the following concentrations:

TABLE 1. Bacterial strains and plasmids

Strain	Description[a]	Source or reference
Escherichia coli		
BW3212	*Δlac-169 Δ(psiF proC phoB phoR)9-6 thi*	B. Wanner
BW3908	*Δlac-169 rpsL thi*	B. Wanner
BW5889	*phoR68 rpsL thi*	B. Wanner
BW6504	*Δlac-169 phoU35 thi*	B. Wanner
BW6718	*Δlac-169 phoM451 phoR68 rpsL thi*	B. Wanner
YMC10 *recA*	*Δlac-169 hsdR hsdM⁺ recA13*	G. Ditta
Vibrio parahaemolyticus		
BB22	Wild type	B. Belas; 3
LM1	Rif^r BB22	This study
LM223	Constitutive mini-Mu *lac* (Tet^r) mutant; Rif^r	This study
LM360	Tet^s LM223(pLM33.12)	This study
LM361	Tet^s LM223(pLM103.25)	This study
LM371	Constitutive mini-Mu *lux* (Tet^r) mutant; Rif^r	This study
LM375	Constitutive mini-Mu *lux* (Tet^r) mutant; Rif^r	This study
LM387	Uninducible mini-Mu *lux* (Tet^r) mutant; Rif^r	This study
LM392	Uninducible mini-Mu *lux* (Tet^r) mutant; Rif^r	This study
LM393	Hemolysin$^-$ mini-Mu *lux* (Tet^r) mutant; Rif^r	This study
LM417	*ompP1::lux* (Tet^r) Rif^r	This study
LM775	Tet^s LM387(pLM33.12)	This study
LM776	Tet^s LM387(pLM103.25)	This study
Plasmids and cosmids		
pACYC177	Ap^r Km^r	12
pLAFRII	Tet^r; pLAFRI with polylinker	F. Ausubel; 19
pLM106.14, -108.7, -111.24, -131.37, and -148.8	Tet^r; overlapping clones with different endpoints of pLAFRII carrying *V. parahaemolyticus ompP*	This study
pLM103.25, -117.18, and -155.10	Tet^r; overlapping clones with different endpoints of pLAFRII carrying *V. parahaemolyticus* phosphate regulation genes	This study
pLM33.12, -154.20, and -157.42	Tet^r; clones with random inserts of *V. parahaemolyticus* DNA into pLAFRII	This study
pLM223	Tet^r Kam^r; pACYC177 with *PstI* insert from LM223	This study
pRK2013	Km^r	G. Ditta; 17

[a] Abbreviations: Rif^r, rifampin resistant; Tet^r or Tet^s, tetracycline resistant or sensitive; Ap^r ampicillin resistant; Km^r, kanamycin resistant; constitutive and uninducible refer to the phosphate regulation phenotype.

kanamycin, 50 μg/ml; rifampin, 100 μg/ml; and tetracycline, 10 μg/ml.

Bacterial matings. Triparental matings were used to promote transfer of cosmids from *E. coli* to *V. parahaemolyticus*. pRK2013 (in strain HB101) served as the helper plasmid for mobilization (17). Matings were performed with overnight cultures by spotting 50 μl of recipient and 100 μl of donor and helper cultures together onto LB plates. After 6 to 12 h of incubation at 30°C, cells were suspended in heart infusion medium and plated on heart infusion agar containing rifampin and tetracycline.

Transposon mutagenesis. Mutagenesis of *V. parahaemolyticus* with mini-Mu *lac* (Tet^r) (11) and mini-Mu *lux* (Tet^r) (15) has been described previously (2, 3). Both transposons generate fusions which couple transcription of the target gene to that of the transposon reporter gene: expression of a *lux* fusion results in the production of light, and expression of a *lac* fusion results in production of β-galactosidase.

Enzyme assays. Alkaline phosphatase activity was assayed and units were calculated by the method of Brickman and Beckwith (10). The substrate was *p*-nitrophenylphosphate (Sigma 104 phosphatase substrate). Phospholipase C assays were performed by the method of Kurioka and Matsuda (28) with *p*-nitrophenylphosphorylcholine (Sigma) as the substrate. Rates of reaction were monitored on a Perkin-Elmer lambda 3A UV/visual light spectrophotometer. After the background rate in the absence of substrate was subtracted, activity was calculated as nanomoles of *p*-nitrophenol (molar extinction coefficient, 1.53×10^4 [28]) produced per minute per unit of optical density at 600 nm (OD_{600}) of culture.

SDS-PAGE. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (1) and visualized by the Fairbanks et al. method (16) of Coomassie blue staining (brilliant blue R; Sigma). The resolving gel was 12.5% acrylamide. Acrylamide and SDS were from BDH (Poole, England).

Preparation of outer membrane fractions. Outer membranes were prepared from 1-ml overnight cultures. Cells were pelleted in an Eppendorf tube in a microcentrifuge. The pellet was suspended in 0.5 ml of 50 mM Tris chloride (pH 8.0)–50 mM EDTA–15% sucrose–0.3 mg of lysozyme per ml and incubated for 30 min on ice. Tubes were centrifuged for 5 min. The pellet was suspended in 1 ml of cold 1% *N*-lauroylsarcosine (sodium salt; Sigma). The detergent selectively solubilized the cytoplasmic membrane (18) of the lysed cells. To reduce viscosity owing to DNA, we passed the sample through a 22-gauge needle. After a 15-min centrifugation, the pellet was suspended in 50 μl of Laemmli sample buffer (29) and boiled for 5 min immediately before 5- to 10-μl samples were loaded on a polyacrylamide gel.

General cloning techniques. Transformations, ligations, and other general cloning procedures were performed by the methods of Maniatis et al. (31). The alkaline lysis quick method (31) for small plasmid preparations worked well for

cosmid preparations, as did large-scale Birnboim-Doly preparations (6). DNA restriction fragments were isolated with an analytical electroeluter (International Biotechnologies, Inc.) following the instructions of the manufacturer. Restriction endonucleases were from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). T4 DNA ligase was from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.).

Cosmid bank construction. High-molecular-weight *V. parahaemolyticus* DNA was isolated from 5-ml cultures by the spooling technique (38; with omission of the freezing step). The DNA was partially cleaved with restriction endonuclease *Sau*3A, phenol extracted, and size fractionated in 10 to 40% sucrose gradients (31). DNA in the 15- to 20-kilobase range was identified by electrophoresis in 0.8% agarose gels. DNA from such fractions was ligated with cosmid vector pLAFRII DNA that had been cleaved with *Bam*HI and treated with calf intestinal phosphatase (Boehringer Mannheim) in an insert/vector molar ratio of approximately 1:3. Ligated DNA was packaged with λ DNA packaging extracts (Amersham Corp., Arlington Heights, Ill.). Recombinant clones were isolated in YMC10 *recA* on LB-tetracycline plates.

DNA hybridization. Southern and colony hybridizations were performed as described by Maniatis et al. (31) with the following specifications. Hybridizations and washes were at 65°C; washes were done in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS; and tRNA (type X; Sigma) was substituted for salmon sperm DNA (0.1 unit per ml of hybridization solution). DNA probes were labeled by nick translation with [α-^{32}P]dCTP and DNA polymerase I from New England Nuclear Corp. (Boston, Mass.) according to a technical bulletin from the manufacturer (20 μCi of labeled dCTP per 0.25 μg of DNA).

Immunological techniques. Antibody production in a New Zealand White rabbit was elicited by lymph node injection of 150 ng of antigen (21). Antigen was prepared by excising a slice containing OmpP from an SDS-polyacrylamide gel and electroeluting the protein into a dialysis bag. The rabbit was boosted with 100 ng of antigen in Freund incomplete adjuvant (Difco) at 4 and 7 weeks. The rabbit was bled at 8 weeks and later. Colony filter blots for immunodetection of OmpP production were made by the method of Meyer et al. (32). Western electrophoretic transfer of proteins (41) after SDS-PAGE was for 2.5 h at 75 V in 0.096 M glycine–0.125 M Tris–20% methanol. Colony filter blots and Western blots (immunoblots) were hybridized with antiserum, washed, and incubated with ^{125}I-labeled *Staphylococcus aureus* protein A as described by Billings et al. (5).

RESULTS

Identification of OmpP. The composition of proteins in the outer membrane was influenced by the concentration of phosphate in the growth medium. Comparison of outer membrane protein profiles of the wild-type strain grown under low- and high-phosphate conditions (Fig. 1, lanes 2 and 3) revealed that under the low-phosphate growth condition a membrane protein, OmpP, of 33.9 kilodaltons (kDa) was synthesized and that a 34.2-kDa protein failed to appear in the outer membrane. Although regulation of the 33.9- and 34.2-kDa proteins appeared to be inversely coordinated, there existed growth conditions such as 2216 medium, in which the phosphate concentration was marginally limiting (approximately 0.6 mM PO_4), in which both proteins were synthesized (Fig. 1, lane 11). Addition of 5 mM K_2HPO_4 to 2216 medium resulted in loss of appearance of OmpP (Fig. 1, lane 12).

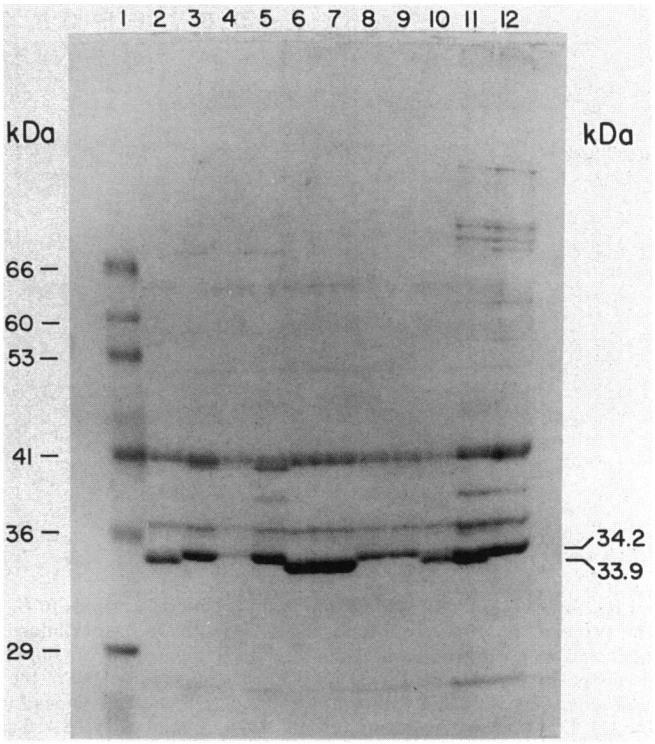

FIG. 1. SDS-PAGE of outer membrane proteins. The following strains were grown in low-phosphate (0.1 mM PO_4) or high-phosphate (5 mM PO_4) minimal marine MOPS or 2216 medium as indicated, and outer membranes were prepared: lane 2, LM1 (low); lane 3, LM1 (high); lane 4, LM417 (low); lane 5, LM417 (high); lane 6, LM375 (high); lane 7, LM371 (high); lane 8, LM392 (low); lane 9, LM387 (low); lane 10, LM776 (low); lane 11, LM1 (2216); lane 12, LM1 (2216 with 5 mM PO_4). In lane 1 are protein standards (from Sigma) with molecular sizes in kilodaltons indicated at the left. In order of decreasing molecular weight they are: bovine serum albumin, catalase, L-glutamic dehydrogenase, alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and carbonic anhydrase.

Cloning of *ompP*. The cloning in *E. coli* of *ompP* was accomplished by immunological techniques. Antibody prepared against *ompP* was used to screen a cosmid library (see Materials and Methods) for production of antigen. Cross-reacting clones were obtained only after the colonies in the bank were grown on low-phosphate medium. Figure 2A shows a Western blot of the five clones obtained from the bank. Antigen was produced only when the cells were grown in low phosphate (lane 7 to 11), and not in phosphate-rich medium (lanes 2 to 5). Some of the clones produced OmpP proteins that had apparent mobilities identical to that of OmpP synthesized in *V. parahaemolyticus* (lane 1), while others produced truncated products. Synthesis of OmpP appeared to be controlled by the *E. coli pho* regulatory system. Figure 2B shows that when OmpP-producing cosmid pLM148.8 was transformed into various *E. coli* phosphate regulation mutants, OmpP failed to be produced in the uninducible strains BW3212 and BW6718 (lanes 7 to 10) and was synthesized constitutively in strain BW6504 (lanes 3 and 4).

Regulation of *ompP*. Mutagenesis with transposon mini-Mu *lux* (15) results in formation of insertion mutations and transcriptional fusions of the promoter of the interrupted gene to the bioluminescence genes (*lux* [14]) carried on the

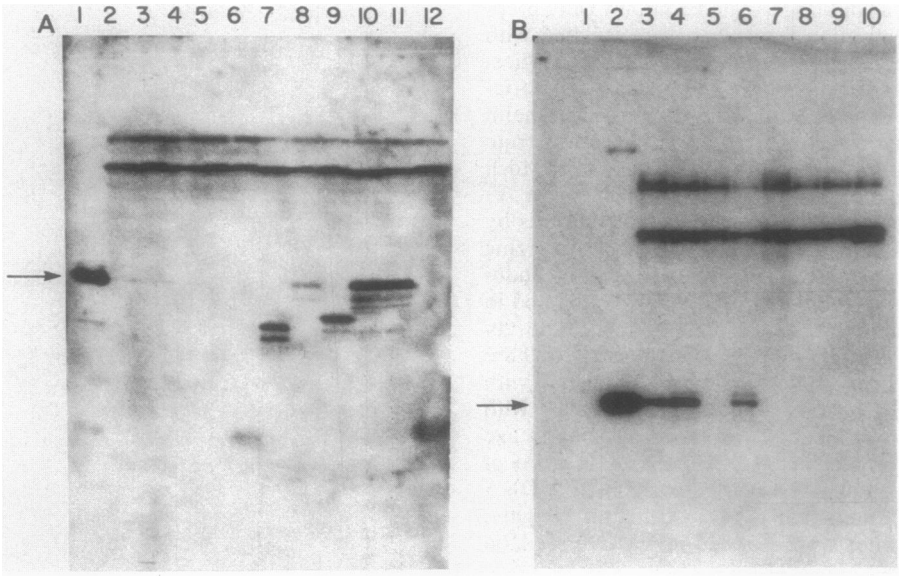

FIG. 2. Western blot analysis of OmpP-producing cosmids in *E. coli*. Whole cells suspended in sample buffer were run on SDS-PAGE. The proteins were electrophoretically transferred to nitrocellulose, incubated with antiserum directed against OmpP, and subsequently incubated with ^{125}I-protein A. Arrows indicate the position of OmpP synthesized in *V. parahaemolyticus*. The samples in panel A were from clones positively identified by colony hybridization screening of the cosmid bank. The cultures of *E. coli* YMC10 *recA* carrying various *V. parahaemolyticus* cosmids were grown in rich (LB) medium (lanes 2 to 6) or low-phosphate MOPS (0.2 mM PO_4, 0.4% glucose) medium (lanes 7 to 12). Lanes: 2 and 7, cosmid pLM106.14; 8, cosmid pLM108.7; 3 and 9, cosmid pLM111.24; 4 and 10, cosmid pLM131.37; 5 and 11, cosmid pLM148.8; 6 and 12, cosmid pLM154.20. Cosmid pLM154.20 was an antigen-negative control. In lane 1 is an outer membrane sample from a *V. parahaemolyticus pho*-constitutive strain (LM223). Panel B contains samples prepared from various *E. coli* phosphate regulatory mutant strains harboring *V. parahaemolyticus* OmpP cosmid pLM148.8. In lanes 3 and 4 the *E. coli* background is BW6504 (constitutive); lanes 5 and 6, BW3908 (wild type); lanes 7 and 8, BW3212 (uninducible); lanes 9 and 10, BW6718 (uninducible). Outer membrane samples from wild-type *V. parahaemolyticus* are in lanes 1 and 2. Strains were grown in high-phosphate (5 mM PO_4; lanes 1, 3, 5, 7, and 9) or low-phosphate (0.2 mM PO_4; lanes 2, 4, 6, 8, and 10) MOPS medium.

transposon. A *V. parahaemolyticus* bank of 10,000 mini-Mu *lux* mutants was screened for operon fusions that showed phosphate-dependent regulation of light production. After the bank was replicated onto low- and high-phosphate 2216 media and allowed to incubate for 24 to 36 h at 30°C, the plates were taken into a darkroom and examined for differential light production on low-phosphate versus high-phosphate plates by both visual inspection and exposure to X-ray film. One hundred phosphate starvation-inducible fusions were identified. Subsequent screening revealed seven mutants defective in the production of OmpP. The outer membrane protein profiles of a representative mutant, LM417, grown in low and high phosphate are shown in lanes 4 and 5, respectively, of Fig. 1. LM417 failed to produce OmpP when grown on low phosphate. Production of the 34.2-kDa outer membrane protein was not affected. Thus, the 34.2-kDa protein is encoded by a gene distinct from *ompP*. With ^{32}P-labeled OmpP-producing cosmid pLM106.14 as the probe, Southern analysis of LM417 confirmed that the transposon insertion mapped in a region homologous to the structural gene (data not shown). Figure 3 shows the induction of light for the *ompP1*::*lux* fusion strain LM417 (panel B) and growth of the culture (panel A) in minimal marine MOPS medium with various concentrations of phosphate. Induction of light occurred simultaneously with the divergence of growth of the culture from the maximal growth rate. Latency of induction was proportional to the amount of phosphate added to the growth medium. Induction of light by phosphate limitation was 500-fold or greater. Since expression of *lux* was transcriptionally coupled to that of *ompP*, we concluded that phosphate limitation resulted in activation of transcription of *ompP*. This conclusion was supported by DNA-RNA hybridization in dot blots which showed that synthesis of *ompP* mRNA increased in response to growth on low phosphate (data not shown).

Porin nature of OmpP. OmpP is a major outer membrane protein of 33.9 kDa. It required boiling in 2% SDS for 5 min for complete denaturation. These properties are similar to those of the pore-forming proteins in *E. coli* (34), and these similarities suggested that OmpP was a porin. Loss of OmpP function in an *ompP*::*lux* fusion strain had no effect on growth rate in limiting inorganic phosphate media (0.05 to 0.5 mM PO_4) compared to the wild-type growth rate. Uptake of the small inorganic phosphate molecules probably does not require induction of a new pore (35). However, the rate of growth in media with large polyphosphates as the phosphate source was affected in the *ompP* mutant. The *ompP* lesion in strain LM417 resulted in considerable impairment of growth in media with linear polyphosphates of chain length 15 and 35 compared with growth of the wild-type strain (Fig. 4).

Other phosphate-regulated gene products. Additional gene products were identified whose synthesis was increased under growth in low-phosphate media: these included phosphatase, phospholipase C, and hemolysin activities. Phosphatase and phospholipase C activities for the wild-type strain (Table 2) increased 45-fold and 8-fold, respectively, when grown under phosphate-limiting (0.5 mM) conditions compared with phosphate-sufficient (5 mM) cultural conditions. Zones of hemolysis (Fig. 5) were produced by the wild-type strain when it was grown on low-phosphate (2216)

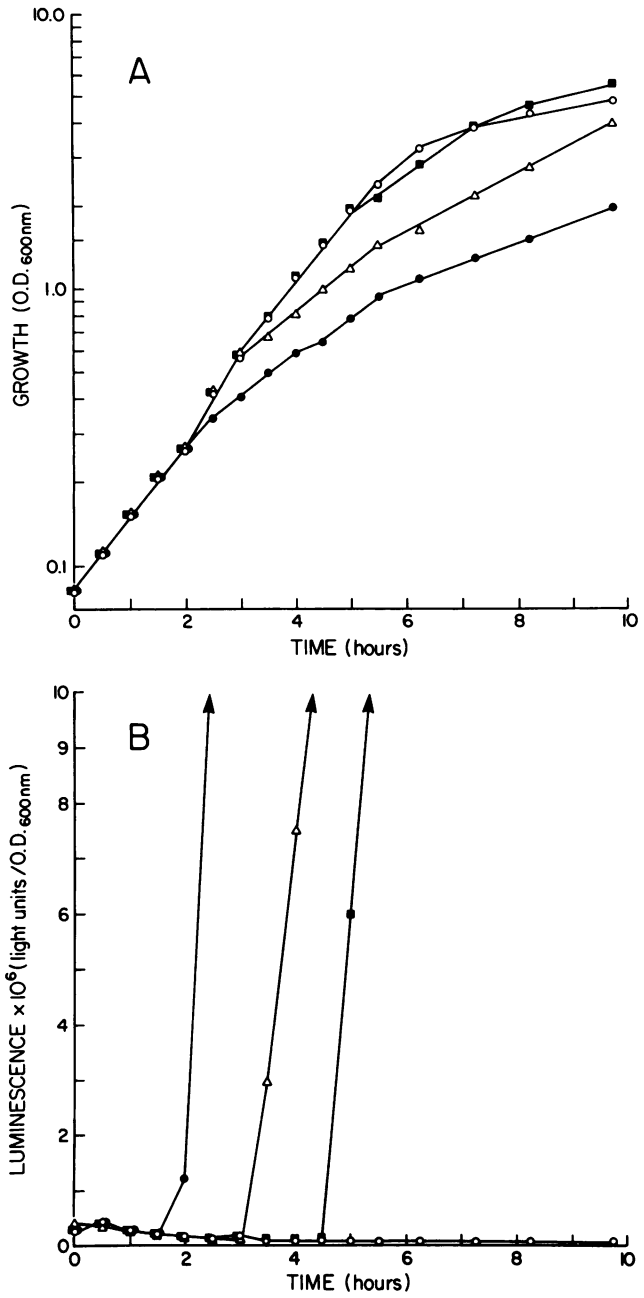

FIG. 3. Growth and induction of luminescence in *ompP*::*lux* fusion strain LM417 in limiting and excess phosphate. An overnight culture of LM417 grown in high-phosphate complete marine MOPS medium was washed in marine MOPS medium with no added phosphate and reinoculated into marine MOPS medium with 0.05 mM PO_4 (●), 0.15 mM PO_4 (△), 0.5 mM PO_4 (■), or 2 mM PO_4 (○). Cultures were incubated at 30°C with aeration. Samples were removed to monitor growth (increase in turbidity at 600 nm, panel A) and luminescence (panel B). Luminescence was measured by counting 100 μl of culture (and dilutions on induction) in an LKB 1211 scintillation counter, using the single photon event or chemiluminescence mode. After background luminescence (100 μl of growth medium) was subtracted, luminescence was expressed as light units (scintillation counts) per minute normalized to 1 ml of culture at an OD_{600} of 1. The arrows in panel B indicate continued increase in luminescence.

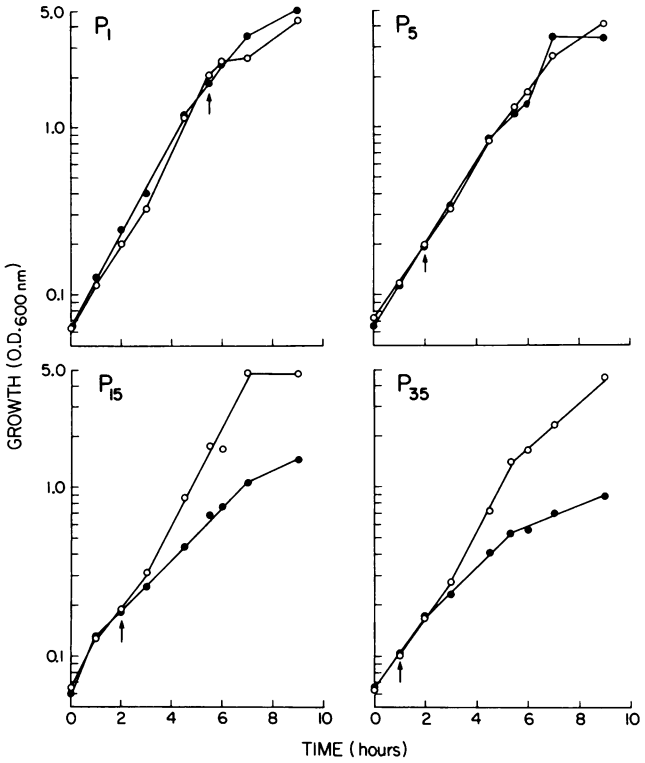

FIG. 4. Growth of wild-type and *ompP* mutant strains on polyphosphates. Overnight cultures of LM1 (○) and *ompP*::*lux* fusion strain LM417 (●) grown in high-phosphate complete marine MOPS medium were washed in marine MOPS medium with no phosphate and reinoculated into minimal marine MOPS medium with 0.5 mM inorganic phosphate (P_1), 0.1 mM polyphosphate of average chain length 5 (P_5), 0.033 mM polyphosphate of average chain length 15 (P_{15}), and 0.015 mM polyphosphate of average chain length 35 (P_{35}). Growth at 30°C with aeration was monitored as increase in turbidity at 600 nm. Time of induction of luminescence in the *ompP*::*lux* strain is indicated by arrows.

medium containing washed sheep erythrocytes (the phosphate in blood is not available before lysis). Supplementation of the medium with 3 mM phosphate repressed production of hemolysin. The hemolysin activity was thermolabile: heating at 60°C for 10 min destroyed activity.

Isolation of phosphate regulation mutants. The hemolysin phenotype was used to identify regulatory mutants. Transposon mutagenesis with tetracycline-encoding derivatives of mini-Mu *lux* and mini-Mu *lac* was used to isolate constitutive and uninducible regulatory mutants that coordinately affected synthesis of the phosphate-regulated gene products. Potential uninducible (and hemolysin structural gene) mutants were identified directly by screening for colonies unable to produce hemolysis on low-phosphate (2216) blood medium. Of approximately 40,000 colonies plated, 20 were hemolysin negative (Hem^-). Candidates for constitutive mutants were identified by transferring 10,000 colonies of a mini-Mu *lux* transposon mutant bank, with a replicator device, onto heart infusion-blood-tetracycline plates, i.e., conditions of high phosphate. Ten colonies produced zones of hemolysis. Figure 5 shows representative mutants (strains 2, 3, and 4).

Approximately half (9 of 20) of the Hem^- mutants were defective in the expression of the other properties known to be under phosphate regulation. Under phosphate-limiting growth conditions these mutants failed to produce OmpP

TABLE 2. Coregulation of alkaline phosphatase and phospholipase C activities in wild-type and mutant strains

Strain	Relevant characteristics	Activity of whole cells[a]			
		Alkaline phosphatase[b]		Phospholipase C[c]	
		Low P	High P	Low P	High P
LM1	Wild type	547	12	2,026	264
LM417	OmpP$^-$	693	14	1,200	ND[d]
LM393	Hem$^-$	552	4	1,560	ND
LM371	Constitutive mutant	1,970	2,484	3,200	2,020
LM375	Constitutive mutant	1,772	2,482	2,580	1,460
LM360	Constitutive mutant, pLM33.12[e]	964	1,809	1,340	860
LM361	Constitutive mutant, pLM103.25[e]	998	47	4,000	200
LM387	Uninducible mutant	2	16	360	460
LM392	Uninducible mutant	4	4	590	290
LM775	Uninducible mutant, pLM33.12[e]	50	24	400	690
LM776	Uninducible mutant, pLM103.25[e]	1,360	42	3,480	160

[a] Overnight cultures grown in low (0.5 mM)- and high (5 mM)-phosphate complete marine MOPS medium were centrifuged and suspended in an appropriate assay buffer.

[b] Alkaline phosphatase activity is expressed as 10^3 A_{420} per minute per OD_{600} unit of culture. Reaction tubes were centrifuged to remove cells before A_{420} measurements.

[c] Phospholipase C activity is expressed as nanomoles of *p*-nitrophenol produced per minute per OD_{600} unit of culture.

[d] ND, Not determined.

[e] pLM103.25 is a recombinant cosmid that carries *V. parahaemolyticus* phosphate regulation genes. pLM33.12 is an unrelated recombinant cosmid.

(representative mutant strains LM392 and LM387 are shown in Fig. 1, lanes 8 and 9) and failed to derepress phosphatase and phospholipase C activity (Table 2). The other Hem$^-$ mutants (11 of 20) resembled the wild type with respect to production of OmpP and phosphatase and phospholipase C activities, and thus remained candidates for transposon insertions in the structural gene for hemolysin. An example of this class of mutant is LM393 (Fig. 5, strain 2; Table 2). All the mutants that produced hemolysin under conditions of phosphate excess also constitutively produced OmpP (strains LM375 and LM371 in Fig. 1, lanes 6 and 7) and phosphatase and phospholipase C (Table 2).

Cloning and complementation of phosphate regulation genes. Chromosomal DNA sequences which flanked the transposon insertions were cloned by using drug resistance as a selectable marker (2). Chromosomal DNA prepared from a mini-Mu *lac* mutant (LM223) of the constitutive phenotype was digested with the restriction endonuclease *Pst*I and ligated with *Pst*I-digested plasmid pACYC177. Selection for tetracycline resistance yielded a hybrid plasmid (pLM223) that contained the left end of mini-Mu *lac*, including the tetracycline and *lac* genes, and bacterial DNA adjacent to the left end of the transposon insertion. Retrieval of clones carrying the intact gene was accomplished by hybridization with the cloned flanking piece. Colony filter blots of the *V. parahaemolyticus* cosmid library were probed with a ^{32}P-labeled nick-translated fragment isolated from a *Pst*I digestion of pLM223. (It was necessary to isolate the fragment for probing because the pACYC177 vector cross-hybridized with the colonies in the bank). Thirteen clones positive for hybridization were detected in the library, which contained 2,500 clones. Some of these cosmids were nick translated and used to probe Southern blots that contained restricted DNA of the insertion mutants with regulatory defects. Insertion of a transposon into a site homologous to the cosmid resulted in perturbation of the hybridization pattern on the Southern blot. Southern analysis (data not shown) revealed that all the mutations resulting in the constitutive phenotype were in a single locus. Furthermore, 9 of the 10 mutations resulting in the uninducible phenotype mapped together, and these insertions were also linked to the mutations conferring the constitutive phenotype. Hybridizing bands may have masked detection of the insertion for the remaining, unmapped uninducible mutant. None of the mutations that were candidates for transposon insertions of mini-Mu *lux* in the structural gene for hemolysin appeared to map in this locus.

Complementation studies in *V. parahaemolyticus* identified cosmids capable of restoring regulatory function to mutant strains with the uninducible and constitutive phenotypes. Transposon-induced regulatory mutants were made tetracycline sensitive by a modified Bochner tetracycline sensitivity selection (9) so that the cosmids of interest could be conjugated into the regulatory mutant strains. On the basis of hemolysin, phosphatase, and phospholipase C activity and OmpP production, cosmids pLM103.25, pLM117.18, and pLM155.10 restored regulation to constitutive and uninducible mutants. Hemolysin production by mutant strains with complementing and noncomplementing control cosmids is shown in Fig. 5. When cosmid pLM103.25 was introduced into an uninducible mutant, the resulting merodiploid strain, LM776, showed phosphate-regulated production of hemolysin, whereas no induction of hemolysin was observed with strain LM775 which carried a control cosmid, pLM33.12. Similarly, for a constitutive mutant, LM223, regulation was restored by cosmid pLM103.25 (merodiploid LM361) and not by pLM33.12 (merodiploid LM360). Table 2 shows that regulation of phosphatase and phospholipase C activity was also restored to constitutive and uninducible mutants by pLM103.25. OmpP was synthesized by merodiploid LM776 when it was grown under

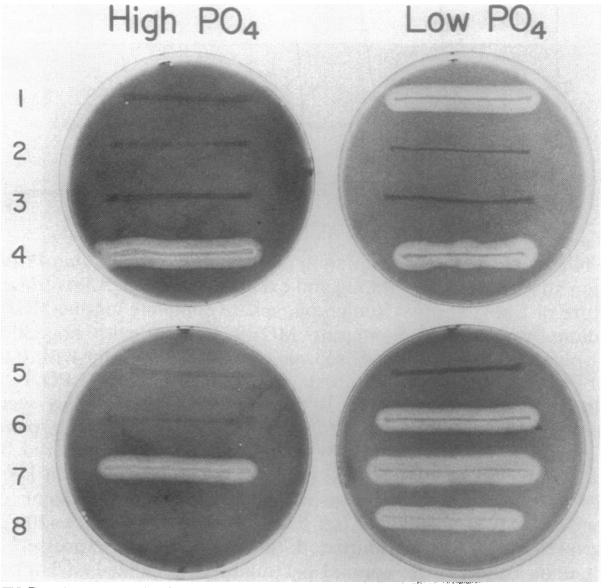

FIG. 5. Hemolytic phenotypes of wild-type, mutant, and merodiploid strains grown on high- and low-phosphate blood media. High PO_4 was 2216 blood medium supplemented with 3 mM PO_4, and low PO_4 was 2216 blood medium. Strain 1 is LM1; 2, LM393; 3, LM387p; 4, LM371; 5, LM775; 6, LM776; 7, LM360; and 8, LM361.

low-phosphate conditions (Fig. 1, lane 10). Transfer of complementing cosmids to mutant *V. parahaemolyticus* strains resulted in regulatory responses similar to those observed in the wild-type strain. However, since the recipient strains were recombination proficient, we cannot exclude the possibility that function was restored not by complementation but by recombinational exchange.

Heterologous complementation studies were done in *E. coli* with well-characterized phosphate regulatory mutants. Cosmids that showed complementing ability in *V. parahaemolyticus*, along with an unrelated control cosmid (pLM157.42), were transformed into the uninducible strain BW3212 [Δ(*phoB phoR*)*9-6*], the constitutive strain BW5889 (*phoR68*), the constitutive strain BW6504 (*phoU35*), and wild-type strain BW3908. Alkaline phosphatase activity was measured for these strains grown under low- and high-phosphate conditions, and the results are shown in Table 3. None of the cosmids affected regulation of alkaline phosphatase in the wild-type strain: derepression of activity was greater than 40-fold. Cosmids pLM117.18 and pLM103.25 allowed full induction of activity for the uninducible strain BW3212. In constitutive strain BW5889 containing these cosmids, alkaline phosphatase was repressed under phosphate-excess growth and was fully derepressed under phosphate-limited growth. Therefore, cosmids pLM117.18 and pLM103.25 contained functional equivalents of *phoB* and *phoR*. Cosmids pLM155.10 and pLM103.25 but not pLM117.18 restored regulation to the constitutive strain BW6504. Therefore, cosmids pLM155.10 and 103.25 compensated for *phoU* function. Cosmid pLM117.18 complemented *phoR* and *phoB* but not *phoU*. Cosmid pLM155.10 provided *phoU* but not *phoR* and *phoB*. Yet pLM103.25 provided all three functions for *E. coli*. Thus, the *phoU*-complementing function was distinct from other *pho* regulatory functions of *V. parahaemolyticus*.

TABLE 3. Complementation of *E. coli* regulatory mutations with *V. parahaemolyticus* cosmids

Strain	Relevant genotype	Cosmid[a]	Alkaline phosphatase activity[b]	
			Low P	High P
BW3908	Wild type	None	358	4
		157.42	328	8
		155.10	350	1
		117.18	336	8
		103.25	450	10
BW3212	Δ(*phoB phoR*)*9-6*	None	1	1
		157.42	1	1
		155.10	1	1
		117.18	500	10
		103.25	375	25
BW5889	*phoR68*	None	282	126
		157.42	142	255
		155.10	200	87
		117.18	1,112	67
		103.25	800	30
BW6504	*phoU35*	None	756	407
		157.42	900	1,100
		155.10	450	20
		117.18	970	600
		103.25	530	24

[a] Plasmid preparations of each culture verified the presence of cosmid.
[b] Overnight cultures were grown in low-phosphate (0.2 mM PO_4) or high-phosphate (5 mM PO_4) MOPS medium supplemented with tetracycline (10 μg/ml) when appropriate. The carbon source was 0.4% glucose, and the medium was supplemented with 0.3 mM proline. Alkaline phosphatase activity of whole cells is expressed as 10^3 A_{420} per minute per OD_{600} unit of culture.

DISCUSSION

We showed in *V. parahaemolyticus* that synthesis of a number of proteins or activities is induced in response to limitation of phosphate in the growth medium. These include an outer membrane porin (OmpP) and phosphatase and phospholipase C activities. Growth defects of a strain lacking OmpP suggest that OmpP plays an important role in the acquisition of large, phosphate-containing molecules. Coordinate induction of a regulon containing genes for a porin protein and enzymes, e.g., phosphatase and phospholipase C, that hydrolyze phosphate-containing molecules would allow a bacterium to adapt to a low-phosphate environment. Using mini-Mu *lux* fusion technology, we isolated many phosphate starvation-inducible fusions, and most of these mutants were not defective in the functions mentioned above. Therefore, members of the phosphate regulon remain to be identified.

In addition, this organism produces a phosphate starvation-inducible hemolysin. Unlike those of *P. aeruginosa* (4, 23), hemolysin and phospholipase C activities of *V. parahaemolyticus* were found to be separate. Mutants lacking hemolysin activity were not affected in the production of phospholipase C. Hemolysin activity has been implicated as an important virulence factor of *V. parahaemolyticus* (7, 30, 36), and a thermostable direct and a thermolabile hemolysin have been cloned (26, 39). Phosphate starvation has not been reported to be necessary to elicit expression of either thermostable direct or thermolabile hemolysin. We suggest that the phosphate starvation-inducible hemolysin represents a distinct hemolysin. We are interested in studying the influence of environmental signals such as phosphate limitation on the expression of virulence gene products. Furthermore, it seems possible that hemolysins may serve more general functions as cytolysins. Phosphate-scavenging potential in the marine environment where nutrients are limiting would be enhanced by secretion of products causing lysis of small marine organisms such as ciliates or flagellates and simultaneous production of proteins capable of extracting phosphate from the cellular contents liberated by the action of cytolysin.

Control of gene expression in response to phosphate limitation requires the function of several master regulatory genes in *E. coli* (for reviews, see references 20 and 40). Mutations that result in the constitutive phenotype in *E. coli* map in two unlinked loci, *phoR* and *pst-phoU*. Mutants with these two classes of defect are phenotypically distinguishable by the extent of expression of alkaline phosphatase (13). Uninducible mutants of *E. coli* result from defects in *phoB*, which is closely linked to *phoR*. In *V. parahaemolyticus* all constitutive mutants were phenotypically identical and contained defects which mapped to one locus. This locus was found to be closely linked to a second locus which contained the sites of insertion of the transposons in the mutants with the uninducible phenotype. Functional similarities between the regulatory systems of both organisms were demonstrated. Expression of the *V. parahaemolyticus ompP* gene responded to phosphate limitation in *E. coli*. Constitutive expression of *ompP* was obtained in an *E. coli* strain with a *phoU* mutation, and expression could not be induced in a host with a *phoB* defect. Therefore, *E. coli pho* regulatory factors regulate a *Vibrio* promoter. Furthermore, complementation of *E. coli phoB*, *phoR*, and *phoU* regulatory

defects was accomplished with cosmids containing the region of DNA encoding the phosphate regulation functions of *V. parahaemolyticus*. Thus, *V. parahaemolyticus* appears to have functions analogous to those in *E. coli*; however, it is also apparent from analysis of mutant phenotypes and from the organization of regulatory loci that there are differences from the *E. coli* system. These differences are intriguing and might be exploited to better understand the genetic mechanism of phosphate regulation. More informative comparison will require the definition of individual phosphate regulation genes of *V. parahaemolyticus*.

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