The nptA Gene of Vibrio cholerae Encodes a Functional Sodium-Dependent Phosphate Cotransporter Homologous to the Type II Cotransporters of Eukaryotes

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The nptA gene of Vibrio cholerae has significant protein sequence homology with type II sodium-dependent phosphate (P) cotransporters found in animals but not previously identified in prokaryotes. The phylogeny of known type II cotransporter sequences indicates that nptA may be either an ancestral gene or a gene acquired from a higher eukaryotic source. The gene was cloned into an expression vector under the control of an inducible promoter and expressed in Escherichia coli. The results demonstrate that nptA encodes a functional protein with activity similar to that of the animal enzyme, catalyzing high-affinity, sodium-dependent P uptake with comparable affinities for both sodium and phosphate ions. Furthermore, the activity of NptA is influenced by pH, again in a manner similar to that of the NaPi-2a subtype of the animal enzyme, although it lacks the corresponding REK motif thought to be responsible for this phenomenon. P uptake activity, a component of which appeared to be sodium dependent, was increased in V. cholerae by phosphate starvation. However, it appears from the use of a reporter gene encoded from the nptA promoter that none of this activity is attributable to the induction of expression from nptA. It is thus proposed that the physiological function of NptA protein may be the rapid uptake of P in preparation for rapid growth in nutrient-rich environments and that it may therefore play a role in establishing infection.

Vibrio cholerae is a gram-negative bacterium that can be isolated from coastal waters around the world. It is a highly varied species, with over 150 identified serotypes. Many strains are pathogenic in humans, usually causing diarrhea. However, only two serotypes (O1 and O139) are known to cause the severe diarrheal disease of epidemic cholera. Owing to the severe and widespread health problems caused by V. cholerae, most investigations have centered upon mechanisms of pathogenicity and efforts to develop an effective vaccine against cholera. Relatively little is known about adaptations of these bacteria to their free-living environment.

In marine and estuarine environments, sodium concentrations are relatively high whereas other essential nutrients can be limiting. One such limitation can be the availability of inorganic phosphate (P), since phosphorus, although relatively abundant in the earth’s crust, often occurs in insoluble forms. Phosphate, however, is an essential nutrient, and therefore bacteria have generally evolved elaborate systems to regulate its uptake and metabolism (36). Two systems are present in bacteria. The Pit system is driven by proton motive force and is characterized by low-affinity, high-capacity uptake. It is active in the presence of normal or increased external phosphate concentrations. The Pst system is activated when the external phosphate level falls below 20 mM (29). Phosphate starvation in Escherichia coli results in the induction of the Pho regulon, which encodes a large number of genes that are distributed around the chromosome and whose expression is controlled by phoB-phoR. PhoR is a histidine kinase which phosphorylates the response regulator PhoB, and this then activates transcription by binding to a specific 18-base consensus sequence which is part of the promoter of genes in the Pho regulon (22, 23).

The Pst operon is a part of the Pho regulon that has been identified both in E. coli and other bacterial species, including V. cholerae (27, 33, 14). It encodes a series of proteins structurally similar to ATP-dependent ABC transporters (2), whose function is the slow but high-affinity uptake of inorganic phosphate (37). The regulon does not, however, contain any genes similar to those for type II sodium-phosphate cotransporters that are central to certain aspects of eukaryote phosphate metabolism or, indeed, any other known sodium-phosphate cotransporters.

The type II cotransporters, first identified in rabbit kidney by expression cloning (39), are encoded by the Npt-2a and Npt-2b genes. The mammalian cotransporter is a transmembrane symporter of approximately 640 amino acids that catalyzes the cellular import of phosphate by utilizing the sodium gradient present across the cytoplasmic membrane. The stoichiometry of transport is approximately three sodium ions per ion of inorganic phosphate. For reviews of mammalian type II cotransporters structure and function see references 5, 8, and 38.

In the present study we demonstrate the presence of a gene immediately downstream of the thyA locus of V. cholerae (GenBank accession no. AJ006514) that encodes a protein involved in sodium-dependent phosphate uptake from the environment that is similar to the type II sodium-phosphate cotransporters described above. Although such proteins exist in various organs of higher eukaryotes, including vertebrates, until now no
equivalent system has been described for prokaryotes. The results suggest either that the vertebrate gene is considerably older than previously thought and may be a development of an ancestral bacterial gene involved in phosphate metabolism or that V. cholerae has acquired a eukaryotic gene by horizontal transfer from a host organism. Whatever its source, V. cholerae has an additional functional phosphate uptake mechanism distinct from that of other prokaryotes so far studied.

**MATERIALS AND METHODS**

**Bacteria and plasmids.** The V. cholerae strain used throughout was JS1569 (32). This strain is a rifampin-resistant derivative of CVD1103, which in turn was derived from the O1 classical strain 569B by deletion of the gene encoding the A subunit of cholera toxin (19).

Cultures of V. cholerae were maintained on agar plates containing a minimal growth medium which was essentially M9 salts (31) supplemented with FeCl$_2$-6H$_2$O (5 mg/liter) and MnCl$_2$-4H$_2$O (4 mg/liter) in which the NaCl concentration was increased to 0.5%. The carbon source was glucose, which was added to a final concentration of 0.2%. The same medium was used in liquid form to grow cultures for use in phosphate uptake experiments.

The E. coli strains used in the present study were HB101 (6) and XL1-Blue (Stratagene, San Diego, Calif.). E. coli strains were maintained on Luria-Bertani agar plates supplemented when necessary with the appropriate antibiotic (ampicillin, 100 μg/ml).

V. cholerae strains tested by PCR for the presence of nptA included a range of clinical and environmental isolates that were both O1 and non-O1 serotypes and toxigenic and nontoxigenic. Strains of other species of Vibrio were taken from the Culture Collection of the University of Göteborg (CCUG). These were Vibrio sp. (non-V. cholerae) strain CCUG 357, V. mettshimkovi CCUG 7409, V. mimicus CCUG 13624, V. alginolyticus CCUG 4990, V. parahaemolyticus CCUG 15657, V. proteus CCUG 3280, V. vulnificus CCUG 13448, and V. fluvialis CCUG 13622.

**DNA manipulation and sequencing.** DNA manipulations, including restriction enzyme analysis, ligations, and transformation were done by standard methods as outlined by Ausubel et al. (3) or according to the instructions supplied by the manufacturers of the reagents used.

The Expand High Fidelity PCR system (Roche Diagnostics Scandinavia AB, Bromma, Sweden) kit was used for generation of the nptA gene with EcoRI and SpeI ends. Chromosomal DNA from V. cholerae JS1569 served as the template for the reaction. The primers used for the PCR of the nptA gene were NAP-1 (5′-CTC-TCT ACC ATC AGC CTC GAA TTC-3′) and NAP-2 (5′-GCG CGG ACT ATG CGT GAC TTG AGT GTG-3′). An additional forward primer (nptA1, 5′-GGG-GGGGATGACAAATGCTACAGCAAAATACGATCAG-3′) was used in conjunction with NAP-2 to amplify a larger fragment carrying the nptA gene and its promoter region. Primers used for detection of nptA in other V. cholerae strains or other Vibrio species were nptA3 (5′-CAG GCC TCG GTG AAG CAA GAC G-3′) and nptA4 (5′-GCC ACT AAA CCA ATC ACA ATC CTG-3′).

All oligonucleotides were synthesized by Synovagen AB (Lund, Sweden). DNA sequencing was performed using an Applied Biosystems 373 automated sequencer with Thermo Sequenase dye terminator cycle sequencing (Amersham Pharmacia Biotech, Solna, Sweden).

**Computer search and similarity algorithms.** Routine computer-aided analysis of DNA sequences was done using the DNA Strider software version 1.3 (24). DNA sequences were assembled in AutoAssembler version 1.4 (Perkin-Elmer Corp., Foster City, Calif.).

DNA database searches were done using the BLAST algorithms (1) and databases available from online services provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The deduced NptA amino acid sequence was aligned with all known sodium-phosphate cotransporter type II sequences by using the online ClustalW (15) service at the European Bioinformatics Institute (www2.ebi.ac.uk/clustalw). Phylogenetic trees were constructed from this alignment by using the Macintosh application TreeView (28).

Transmembrane regions and protein topology of NptA and human NaPi-3a were predicted using online services. NptA was analyzed by TopPred2 (34) in the prokaryotic mode at the Department of Biochemistry, Stockholm University, Stockholm, Sweden (http://www.biokemi.su.se/), and NaPi-3a was analyzed by TopPred2 in the eukaryotic mode. TopPred2 did not detect the membrane-associated hinge region III, but the presence of this region was inferred from existing models and predicted by Tmpred (Expasy [www.expasy.ch]).

Statistics and curve fitting were performed using the Prism software package (GraphPad, San Diego, Calif.).

**Sodium-dependent phosphate uptake by V. cholerae and E. coli.** V. cholerae strain JS1569 was grown overnight at 30°C in minimal medium in the presence of phosphate. The culture was divided into two equal aliquots. The cells were spun down (6,000 × g for 20 min), and half of the pellet was resuspended in an equal volume of fresh medium containing phosphate. The other half was resuspended in medium buffered with Tris-HCl (20 mM) containing no phosphate. The two cultures were then incubated with shaking at 30°C for a further 3 h. The cells were collected by centrifugation, washed twice, and finally resuspended in 1 ml of buffer (10 mM Tris-HCl, pH 7.5) containing 1 mM MgCl$_2$ and 1 mM CaCl$_2$. Volumes were finally adjusted so that the optical densities of different cell suspensions used in each experiment were the same.

Cells were incubated at 30°C in 10 mM Tris-HCl (pH 7.5) with 10 μCi of $^{32}$P-labeled inorganic phosphate (Amersham) per ml adjusted with cold K$_2$HPO$_4$ to a final concentration in the reaction mixture of 1 mM, in the presence and absence of NaCl which was added to a final concentration of 100 mM. When NaCl was absent, the osmolarity was preserved by addition of choline chloride to the same concentration. The cells were removed from the reaction mixture by centrifugation through a nonaqueous layer containing a 4:1 mixture of n-butyl phthalate and corn oil (18). The pellet was resuspended in a small volume of 10 mM Tris-HCl (pH 7.5) containing 1 mM MgCl$_2$ and 1 mM CaCl$_2$, and the amount of $^{32}$P-labeled phosphate accumulated in the pelleted cells was determined in a scintillation counter.

Cultures of E. coli strain HB101 carrying the nptA gene on a recombinant plasmid were treated in essentially the same manner after harvesting. However, the cells were grown up overnight in Luria-Bertani broth at 37°C. Five hundred microliters of the overnight culture was used to inoculate 50 ml of fresh medium, and the new culture was grown at 37°C for 2 to 2 1/2 h. The culture was then divided into two 25-ml aliquots, and IPTG (isopropyl-β-d-thiogalactopyranoside) was then added to one aliquot to a final concentration of 1 mM. The second aliquot was used as a noninduced control. Incubation was continued for a further 2 1/2 to 3 h before the cells were harvested by centrifugation, washed, and resuspended in 10 mM Tris-HCl (pH 7.5) containing 1 mM CaCl$_2$ and 1 mM MgCl$_2$.

For kinetic studies on NptA protein expressed in E. coli, essentially the same methods were used. However, the amount of phosphate in the reactions was varied by varying the amount of cold P$_i$ added, whereas the amount of labeled P$_i$ was kept constant. In reactions where the influence of Na$^+$ concentration on P$_i$ uptake was measured, the amount of P$_i$ was kept constant (1 mM) and the amount of Na$^+$ added to the reaction mixtures was varied, with the addition of choline chloride to preserve osmolarity.

To determine the activity of NptA at different pH values, similar assays were done in which the reactions were buffered in the range from pH 6.5 to 9.0. To assess the dependence of NptA activity on the maintenance of a sodium concentration gradient across the cell membrane, the sodium ionophore monensin was added to the uptake assay mixture described above in the concentration range from 0 to 100 μM.

Reactions in all cases were run for a constant time (4 min). All reactions were done in quadruplicate.

Protein concentrations were determined using the MicroBCA kit (Pierce) and used to normalize isotope uptake measurements in the different samples.

**CZO assays.** Assays of catechol-2,3-dioxygenase (C23O) were done essentially by the spectrophotometric assay described by Ingram et al. (17). Cells in which activity was measured were harvested by centrifugation and resuspended in a volume of cold 100 mM sodium phosphate buffer (pH 7.5) containing 10% (vol/vol) acetone, such that the cells were concentrated at least 10 times with respect to the original culture volume. All subsequent procedures were done at 4°C or on ice. The cells were disrupted by sonication, and the resulting extract was centrifuged at 13,000 × g for 10 min in order to remove cell debris. Extracts were used immediately. Assays were done at room temperature in a standard 3-ml reaction volume containing 100 μl of cell extract and 0.2 mM catechol (Sigma) in 100 mM sodium phosphate buffer (pH 7.5). Reactions were started after a 1-min equilibration period by the addition of catechol (30 μl of a freshly made 20 mM stock solution). The change in absorbance at 375 nm resulting from the formation of 2-hydroxymuconic semialdehyde was monitored for 5 min. Activities were expressed as milliunits per milligram of protein as described by Sala-Trepat and Evans (30). The protein concentrations in the samples were determined as described above.

**Nucleotide sequence accession number.** The sequence of the nptA gene has been deposited in the EMBL database under accession number AJ010968.
RESULTS

Cloning of the \textit{nptA} gene, encoding the sodium-dependent phosphate pump of \textit{V. cholerae}. As a part of the characterization of the \textit{lng-thyA} locus of \textit{V. cholerae}, 600 bp of DNA downstream of the \textit{thyA} gene was sequenced. A large, incomplete open reading frame reading in the opposite orientation to \textit{thyA} was detected, which, when compared with sequences in the GenBank database, was found to have significant homology with a class of renal sodium-dependent phosphate transport proteins (NaPi-2) from a variety of vertebrates, including humans. In order to obtain the missing amino-terminal sequence of the protein, a \textit{HindIII} digest of \textit{V. cholerae} chromosomal DNA was hybridized against a \textit{PstI}/\textit{HindIII} probe derived from the sequence already obtained. A single 1.5-kb band appeared, and this was subsequently isolated and cloned in pBR322.

The cloned fragment was sequenced, and a single open reading frame encoding a protein of 382 amino acids was identified. The TAA stop signal is located 18 bases upstream of the 18-base perfect inverted repeat that we had previously proposed as the transcription terminator for the \textit{thyA} gene, which is transcribed in the opposite direction (GenBank accession no. AJ006514). Owing to its homology with vertebrate type II sodium-dependent phosphate transport protein (NaPi-2) genes, the gene was named Na\textsuperscript{+}-dependent phosphate transporter A, or \textit{nptA}. Sequence similarity searches using the BLAST program revealed that the DNA immediately upstream of the \textit{nptA} gene carries a homologue of the \textit{nhaR} gene, encoding the Na\textsuperscript{+}/H\textsuperscript{+} antiporter-affected protein (NhaA) regulator reading in the opposite direction (41). The position of the gene relative to \textit{thyA} and \textit{nhaR} is shown in Fig. 1.

**Occurrence of \textit{nptA} in \textit{Vibrio} species.** In order to determine how widespread the occurrence of the \textit{nptA} gene is within \textit{Vibrio} species, strains of \textit{V. cholerae} from different clinical and environmental sources and samples of other different \textit{Vibrio} species were subjected to PCR amplification with \textit{nptA}-specific primers (see Materials and Methods). Within \textit{V. cholerae}, comparison of our deposited sequence (from an O1 classical strain) and that of the entire genome (from an O1 El Tor strain) showed not only that the gene is present but also that it is highly conserved. PCR analysis of toxigenic and nontoxigenic strains of \textit{V. cholerae} from different serotypes confirmed that the gene was present in all of the strains tested, giving an amplified band of 279 bp. A range of other \textit{Vibrio} species was tested with the same primers. All except one (identified only as a non-\textit{V. cholerae} \textit{Vibrio} species, strain CCUG 537) gave negative results, demonstrating that the gene may not be widely distributed in other \textit{Vibrio} species (data not shown).

**Structural comparison with other type II sodium-phosphate cotransporters.** Alignment of the deduced NptA amino acid sequence with known eukaryotic sequences showed a high degree of sequence similarity between NptA and previously identified NaPi-2 cotransporters. An alignment of the deduced NptA amino acid sequence with the NaPi-3 (human) sequence is shown in Fig. 2A. NptA and mammalian proteins have a similar topology with the exception of deletions in the N terminus, the central loop of the protein, and the C terminus. The absence of the last transmembrane region from the NptA sequence suggests that the bacterial C terminus is located extracellularly, as opposed to intracellularly in animal transporters (Fig. 2B).

A phylogenetic analysis of representative type II cotransporter sequences is shown as a rectangular cladogram in Fig. 3. It can be seen that NptA occupies a separate branch in the phylogenetic tree, suggesting a significant evolutionary dis-
tance between it and other known NaPi-2 sequences, and the divergence of NptA and other type II cotransporters probably predates the divergence of type IIa and type IIb cotransporters.

Enhanced sodium-dependent Pi uptake in *V. cholerae* is induced by phosphate starvation. *V. cholerae* cultures were shown to be able to take up 32P-labeled phosphate significantly more rapidly and to a larger extent after incubation under conditions of phosphate starvation than after incubation in the presence of phosphate. It is thus clear that there are inducible phosphate uptake mechanisms in *V. cholerae*. Despite increased sodium-independent phosphate uptake, suggesting the presence of more than one inducible system in the cells, uptake was shown to be significantly enhanced by the addition of sodium to cells grown in the absence of phosphate, compared to cells grown with phosphate (Fig. 4).

**nptA** expression is not induced by Pi limitation. In order to determine whether any of the observed increase in Pi uptake could be attributed to **nptA** expression, a low-copy-number plasmid based on the p15A origin of replication was constructed, which carried the **nptA** gene together with the entire intergenic region between **nhaR** and **nptA**, which is assumed to carry the **nptA** promoter. A promoterless **dmpB** gene, encoding C23O (4), was inserted into the XbaI site within the **nptA** gene such that it was under the control of the **nptA** promoter (Fig. 1). The resulting strain was then subjected to growth on minimal medium in the presence and absence of Pi as described in Materials and Methods. The cells were then harvested, disrupted by sonication, and assayed for **dmpB** expression as indicated by the presence of C23O as described in Materials and Methods. Following growth under these conditions, no C23O activity could be detected regardless of the concentration of Pi in the growth medium. The presence of the plasmid was confirmed by plasmid isolation from cultures used in the assays. This suggests that **nptA** is not
induced in response to alterations in external $P_i$ concentration and that the observed increases in $P_i$ uptake resulting from $P_i$ starvation are not attributable to nptA expression.

**Activity of the cloned nptA gene product in E. coli.** In order to confirm the activity of the nptA gene product, chromosomal DNA from *V. cholerae* NptA (accession no. CAA09443) and *Caenorhabditis elegans* NaPi cotransporter (AAA81148), human NaPi-IIa and NaPi-IIb (AAC36354 and AAC98695, respectively), mouse NaPi-IIa and NaPi-IIb (AAC42026 and AAC80007, respectively), rat NaPi-IIa (AAC37608), rabbit NaPi-IIa (I46534), sheep NaPi-IIa (CAA04715), opossum NaPi-IIa (AAA30978), bovine NaPi-IIb (CAA57345), Xenopus laevis NaPi-IIb (AA21134), zebrafish NaPi-IIb (AF297180), flounder NaPi-IIb (AAAB16821), trout NaPi-IIb (AF297186), and shark NaPi-IIb (AF297182).

**DISCUSSION**

The present work describes the cloning and characterization of the nptA gene from *V. cholerae*, encoding a type II sodium-dependent phosphate transport protein (NaPi-2) previously identified only in eukaryotes. In higher vertebrates these proteins function in the initial uptake of phosphate in the small intestine and reabsorption of inorganic phosphate from excreted fluid in the kidneys. Expression of the cloned nptA gene in *E. coli* clearly demonstrates that the gene product is a functional $Na^+\cdot P_i$ cotransporter catalyzing the sodium-dependent uptake of $P_i$. The striking similarity in structure between the eukaryotic and prokaryotic proteins reflects a similarity in function with respect to...
the uptake of phosphate and sodium ions and the requirement for a sodium gradient for activity.

Overexpression of the cloned nptA gene in E. coli repressed the background Pᵢ uptake observed in noninduced cells, presumably due to the accumulation of high intracellular phosphate concentrations resulting from nptA expression during growth in phosphate-rich medium. This indicated immediately that the NptA protein was able to profoundly affect the Pᵢ balance of cells in which it was expressed. A low background in induced cells, together with improved methodology for the rapid removal of cells from reaction mixtures, allowed the accurate and reproducible measurement of NptA-driven phosphate uptake under different conditions. The results of these experiments indicate that the apparent Kᵢₘ values of the NptA protein with respect to Pᵢ and Na⁺ (300 μM and 75 mM, respectively) are comparable to but slightly higher than those obtained for similar eukaryotic NaPi-2 proteins representing the two types (α and β) expressed in Xenopus oocytes, i.e., 30 to 250 μM for Pᵢ and 35 to 60 mM for Na⁺ (7, 9, 12, 13, 16, 11, 26).

With respect to the observed pH dependence of the NptA protein, there was a reduction in activity at low pH similar to that seen in NaPi-2a proteins from higher organisms although this characteristic does not correlate with the pH₇₅7REK sequence thought to be responsible for proton sensitivity in these proteins (10). Overall these results indicate that NptA is a sodium-dependent phosphate cotransporter sharing many characteristics with the animal NaPi-2a cotransporter. However, detailed kinetic and electrophysiological characterization of NptA would require patch-clamp studies in a model system such as Xenopus oocytes (8); such investigations are ongoing.

The V. cholerae amino acid sequence has an overall identity of 33% to the human sequence, with an overall homology of 50% (38 and 58%, respectively, if one excludes the large deletions). Comparison with the overall identity between rat type II and III sodium-phosphate co-transporters, which is only 15% despite the fact that they share a similar function (21), makes this level of similarity all the more striking.

Structural predictions suggest that the NptA protein and mammalian transporters share similar protein architecture, with the exception of the possible absence of the last transmembrane helix and the concomitant change in C-terminal orientation (Fig. 2B). However, computerized predictions of protein topology are putative, and the orientation of the extracellular loops in NptA must be confirmed experimentally. Nonetheless, it is noteworthy that all major deletions in the V. cholerae protein occur either at the C terminus, deleting the last transmembrane helix, or in the N-terminal part of the extracellular loop between membrane-spanning domains III and IV (21). In eukaryotes this loop is heavily glycosylated and probably has regulatory functions that are absent from the V. cholerae protein. The topological model presented in Fig. 2B is based on the model of the mammalian cotransporter as presented by Werner and Kinne (38). The hinge regions III to HIV have some helical characteristics, and if taken as transmembrane helices, they would produce a quite different topology. In Fig. 2B they are assigned as membrane-associated regions in accordance with the model of the animal cotransporter.

BLAST searches revealed that the similarity between the higher eukaryotic Npt-2a genes and nptA is much greater than similarities between nptA and bacterial genes for which scores were recorded. It can be concluded that the Npt-2a and nptA genes are closely related and have probably not arisen as a result of convergent evolution. Phylogenetic analysis, however, suggests that there is a considerable evolutionary distance between them (Fig. 3), raising the possibility that nptA might be an ancestral gene.

Preliminary results of PCR analysis of strains from a range of other Vibrio species that exist in similar environments suggest that nptA is confined to V. cholerae and may potentially be a diagnostic feature that is independent of other virulence
markers. This together with its similarity to eukaryotic Npt-2 genes and the absence of similar genes in other sequenced bacterial chromosomes raises the alternative possibility that *V. cholerae* has acquired the gene from a higher eukaryotic source. This has recently been noted for other bacteria with either symbiotic or pathogenic associations with plants or animals (20, 40). Arguing against this, however, are the G/C content (47.9% for *nptA* compared to 47.7% for chromosome I [14]) and the codon usage in the *nptA* gene, which are consistent with those of the *V. cholerae* chromosome I as a whole. Significantly, in *V. cholerae* the *nhaR* gene, encoding the NhaA Na+/H+ antiporter-affecting protein regulator, was found to be located immediately upstream of the *nptA* gene, reading in the opposite direction. It appears that the two genes are linked in some way, with preliminary results suggesting that the *nhaR* gene may have a role in the regulation of *nptA* expression (M. Lebens et al., unpublished data). Furthermore, repeated sequence motifs reminiscent of the *E. coli* phosphate (Pho) box consensus within the intergenic region between *nhaR* and *nptA* suggest that despite the absence of an *nptA*-like gene from other bacteria, *phoRB* may be involved in controlling expression of one or both of the genes. Indeed, the promoter structures for the *phoB* and *phoR* genes (35) (accession number AF043352) and for the *pstS* homologue retrieved from the *V. cholerae* genome have sequence similarities with the Pho box structures upstream of *nptA* (data not shown). The exact mechanisms involved in regulation of *nptA* expression are currently under investigation but appear to be complex. Their elucidation will help to shed light on the physiological role of *nptA* in *V. cholerae*, which remains unclear.

The recent publication of the entire *V. cholerae* genome (14) reveals that Pit and Pst homologues are present. These both
have a much higher affinity for \( P_i \) than does NptA, with apparent \( K_m \)s of between 25 and 38 \( \mu \)M for the constitutive Pit system (42) and 0.2 \( \mu \)M for the inducible Pst system (29, 42). Furthermore, we have demonstrated that in \emph{V. cholerae}, similar to in other bacteria, the uptake of phosphate is highly up-regulated when cells are grown under conditions of phosphate starvation. In contrast to \emph{E. coli}, \emph{V. cholerae} exhibited a weak sodium-dependent \( P_i \) uptake after growth in minimal medium. This uptake was enhanced 2.5 times by phosphate starvation. However, reporter gene studies failed to show any induction of \( nptA \) transcription under these conditions. This suggests that the enhanced sodium-dependent \( P_i \) uptake measured was due not to an activation of the \( nptA \) gene but to some other process by which Pit and Pst activities are enhanced by an increase in the potential of the sodium gradient across the cell membrane.

\( nptA \) may have a role in the pathogenicity of \emph{V. cholerae}, as it colonizes the human intestine. \emph{V. cholerae} is the only \emph{Vibrio} species that gives rise to epidemic cholera. The changes as it passes from its water environment to the human intestine are profound and include sudden changes in pH and the availability of both energy and phosphate. It is thus possible that NptA functions as a low-affinity, high-capacity cotransporter, possibly meeting the cells’ need for large amounts of \( P_i \) during rapid growth in nutrient-rich environments. \( nptA \) may thus have a
role in the cascade of events that lead to the establishment of the infection that distinguishes *V. cholerae* from other vibrios, which, even when carrying the same virulence factors, do not give rise to such devastating epidemics. Alternatively, responses to levels of phosphate in the environment may influence the ability of strains to become infectious. In the absence of any investigation of phosphate metabolism in *V. cholerae* and given the obvious differences from other gram-negative organisms highlighted by the present findings, these questions remain to be answered.

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