

Inorganic Polyphosphate in *Vibrio cholerae*: Genetic, Biochemical, and Physiologic Features

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***Vibrio cholerae* O1, biotype El Tor, accumulates inorganic polyphosphate (poly P) principally as large clusters of granules. Poly P kinase (PPK), the enzyme that synthesizes poly P from ATP, is encoded by the *ppk* gene, which has been cloned from *V. cholerae*, overexpressed, and knocked out by insertion-deletion mutagenesis. The predicted amino acid sequence of PPK is 701 residues (81.6 kDa), with 64% identity to that of *Escherichia coli*, which it resembles biochemically. As in *E. coli*, *ppk* is part of an operon with *ppx*, the gene that encodes exopolyphosphatase (PPX). However, unlike in *E. coli*, PPX activity was not detected in cell extracts of wild-type *V. cholerae*. The *ppk* null mutant of *V. cholerae* has diminished adaptation to high concentrations of calcium in the medium as well as motility and abiotic surface attachment.**

Inorganic polyphosphate poly P is a linear polymer of up to hundreds of orthophosphate (P_i) residues linked by high-energy phosphoanhydride bonds. Among known functions, poly P can serve as a substitute for ATP in kinase reactions, a P_i reservoir, and a chelator of divalent metals (9). Poly P is ubiquitous in nature, having been found in all organisms examined (15), yet little is known about its physiological roles (14).

Several poly P-metabolizing enzymes have been purified, and the genes encoding them have been cloned (14, 28). The enzyme primarily responsible for poly P synthesis in *Escherichia coli* is poly P kinase (PPK), which catalyzes the polymerization of the γ phosphate of ATP into a poly P chain (1). Poly P can be hydrolyzed to P_i by an exopolyphosphatase (PPX) (3). In *E. coli*, the encoding genes, *ppk* and *ppx*, respectively, form an operon. The inability to accumulate poly P upon deletion of this operon or upon the overproduction of PPX has produced several striking phenotypes in *E. coli* (6, 20, 25): decreased long-term survival in stationary phase; increased sensitivity to oxidative, osmotic, and thermal stresses; and defects in adaptive growth in minimal medium after a shift from rich medium. These phenotypes are likely due to the decreased expression of the *rpoS* gene, which encodes the principal stationary-phase sigma factor, σ^S , or RpoS (25). These and related results (4) suggest that poly P is an effector signal for responses to acute stringencies and adaptations in the stationary phase.

Recently available genome sequences have revealed that PPK is highly conserved in many bacterial species, including some important pathogens (26). This also implies that PPK and/or poly P has fundamental physiological roles in bacteria. The *ppk* knockout mutant of *Pseudomonas aeruginosa* PAO1 shows a dramatic deficiency in motility, both flagellar and pilus mediated, an inability to form biofilms, and a loss of virulence (22, 23, 24). *ppk* null mutants of several other pathogens and of *E. coli* also exhibit reduced motility and reduced abiotic surface attachment (22, 24).

Vibrio spp. are among the most common microorganisms in environmental surface waters, such as lakes and rivers. *Vibrio*

cholerae O1 is an enteropathogenic gram-negative bacterium that causes severe diarrheal disease. An *rpoS* mutant of *V. cholerae* (29) revealed that RpoS is required for *V. cholerae* persistence in a medium devised to simulate natural aquatic habitats. A gene highly homologous to *E. coli ppk* was found in the *V. cholerae* database of The Institute for Genomic Research (TIGR) (26). The essential role of poly P for the stationary-phase survival of *E. coli* and the possibility of a similar role in *V. cholerae* prompted us to study its PPK and to examine the phenotype of a *ppk* knockout mutant.

V. cholerae O1, biotype El Tor, accumulates much higher levels of poly P than *E. coli* under normal growth conditions. High accumulations of poly P are stored as granules following a shift from a defined medium lacking P_i to one with an excess (20 mM). The *ppk* null mutant is defective in motility and abiotic surface attachment and fails to adapt to high concentrations of calcium.

MATERIALS AND METHODS

Strains and plasmids. *E. coli* strains MG1655 ($\lambda^- F^-$) and CF5802 (MG1655 Δ ppk Δ ppx::Kan [17]) were the wild-type and mutant strains, respectively. Recombinant plasmids based on pBluescript II KS(+) and SK(+) (Stratagene, La Jolla, Calif.) were prepared from DH5 α transformants. Suicide plasmids based on pKNG101 (11) were replicated in *E. coli* strain S17-1(λ pir) (29); pFZY1 (13) was used as a low-copy-number vector. The 92A1552-Rif^r wild-type strain of *V. cholerae* O1 (El Tor, Inaba [29]) and the cosmid library of its genomic DNA were provided by F. Yildiz, Department of Microbiology and Immunology, Stanford University.

Media and growth conditions. A MOPS (morpholinepropanesulfonic acid)-buffered minimal medium (21) was used to impose P_i -limiting conditions for the growth of *V. cholerae*. Media were supplemented with ampicillin (100 μ g/ml), kanamycin (150 μ g/ml), or streptomycin (100 μ g/ml) to select for antibiotic-resistant transformants of *V. cholerae*.

Plasmid construction. The *V. cholerae* PPK sequence was obtained by a BLAST search of the TIGR genome sequence database using the *E. coli* sequence. To obtain the *ppk* region of *V. cholerae*, two PCR primers were designed: VCPPKFOR1, CCTTCTAGACAACCTCTATGACACTAAAGGCAC, and VCPPKREV1, CCTGTCTGACTCTGCCGATGAGATAAAGAC. VCPPKFOR1 contains an *Xba*I site, and VCPPKREV1 contains a *Sal*I site, each located at the 5' end (underlined). These primers yielded a 1.95-kb PCR product using genomic DNA prepared from the wild-type *V. cholerae* strain 92A1552-Rif^r as a template. This fragment begins at position -106 relative to the A in the start codon at +1 and ends at position +1829. After digestion with both *Xba*I and *Sal*I, this PCR product was cloned into *Xba*I- and *Sal*I-digested pBluescript II KS(+); the resulting plasmid was designated pVCK1.

The cosmid pVCK20 was obtained by colony hybridization from a *V. cholerae* genome library using the 1.95-kb PCR fragment as a probe; pVCK20 contained an insert of more than 40 kb in which the *ppk* homologous region was limited to a 2.5-kb *Nco*I-*Bgl*II fragment (pVCK35 [Fig. 1A]).

Plasmids containing the cloned *V. cholerae ppk* gene were constructed as

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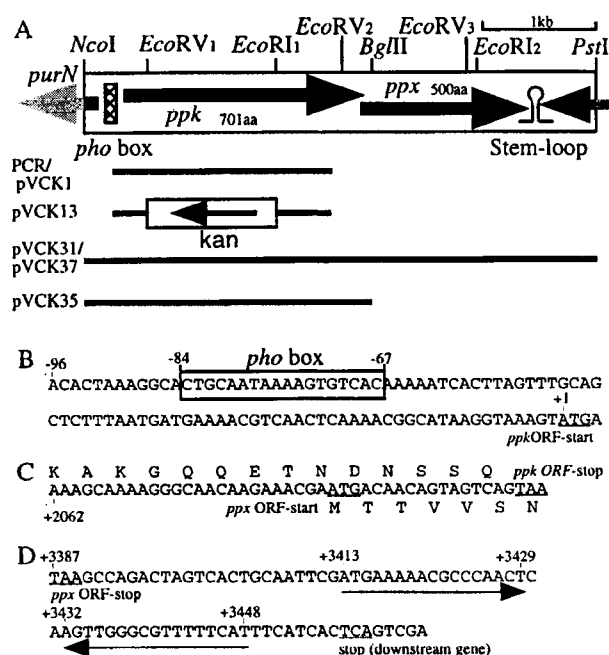


FIG. 1. The *ppk ppx* operon in *V. cholerae* and recombinant plasmids. (A) The large box represents the cloned and sequenced 4-kb *NcoI-PstI* chromosomal fragment containing the operon. ORFs are indicated by arrows. The lines under the box indicate the portions of the *ppk ppx* region inserted in the plasmids indicated to the left. The nonunique *EcoRI* and *EcoRV* restriction sites in the 4-kb *NcoI-PstI* fragment are denoted by subscripts. (B) Putative *pho* box sequence in the *ppk* promoter region. (C) Sequence overlap between the *ppk* and *ppx* ORFs. The center sequence is the nucleotide sequence of the *ppk ppx* ORF junction, and the top and bottom sequences are the deduced amino acid sequences for PPK and PPX, respectively. (D) Putative transcriptional terminator sequence in the region downstream of *ppx*. The pair of sequences indicated by the arrows have the potential to form a stem-loop structure. The underlined nucleotides are the indicated start and stop codons.

follows. A 6.5-kb *HindIII-PstI* fragment containing *ppk* prepared from pVCK20 was inserted into similarly restricted pBluescript II SK(+). The resulting plasmid, pVCK27, was digested with *HindIII* and *NcoI*, and the 7-kb fragment was isolated. After the ends were filled in, this fragment was self-ligated. The resulting plasmid, pVCK31 (Fig. 1A), contains the 4-kb *NcoI-PstI* fragment in pBluescript II SK(+). A smaller plasmid, pVCK35 (Fig. 1A), was constructed by digestion of pVCK31 with *BglIII* and *BamHI*, followed by self-ligation of the resulting longer fragment; pVCK35 harbors the 2.5-kb *NcoI-BglIII* fragment in pBluescript II SK(+).

A low-copy-number plasmid containing the *V. cholerae ppk ppx* loci, pVCK37, was constructed as follows. pVCK31 was digested with *BamHI* and *Sall*, and the fragment with the 4-kb *NcoI-PstI ppk ppx* region was isolated. The *BamHI-Sall* fragment of about 4 kb was inserted into a similarly restricted low-copy-number vector, pFZY1, resulting in plasmid pVCK37.

Construction of the *V. cholerae ppk* knockout mutant. To make the deletion-insertion mutation allele of *ppk*, a 1.2-kb *EcoRV* (site 1)-*EcoRI* (site 1) region of pVCK1 (Fig. 1A) was replaced with a 1.3-kb *HincII-EcoRI* fragment containing the kanamycin cassette from pVCK4, which was constructed by insertion of a 1.3-kb *HincII-HincII* fragment with the cassette from pUC4K (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.) into the *EcoRV* site of pBluescript II SK(+). From the resulting plasmid, pVCK7, the 2.1-kb *XbaI-Sall ppk::Kan* fragment was transferred into the suicide vector pKNG101, and the resulting plasmid was designated pVCK13 (Fig. 1A).

pVCK13-integrated transformants (single-crossover recombinants) of the *V. cholerae* wild-type strain 92A1552-Rif^r which exhibited both kanamycin and streptomycin resistance phenotypes were obtained. After two successive overnight cultivations of the integrants in Luria broth (LB) supplemented with 5% sucrose, the *ppk* knockout mutants (double-crossover recombinants) were obtained as kanamycin-resistant but streptomycin-sensitive colonies. The chromosomal mutations were confirmed by Southern blot analysis, and one of the resultant recombinants, KVC3, was selected as the *ppk* knockout mutant of *V. cholerae*.

Biochemical assays. Cell extracts from *V. cholerae* were prepared as from *E. coli* (16), modified only by a 2-min sonication after lysozyme treatment. PPK and PPX activities were assayed as described previously (1, 17). Poly P levels were determined by the nonradioactive method (4). The cloned DNA fragment was sequenced by the PAN Facility.

Electron microscopy. Strains were cultivated as described in the legend to Fig. 3D. Cells were harvested at zero time and 2 h after the addition of P_i and were washed three times with 0.9% sodium chloride (zero time) or phosphate-buffered saline (2 h) solution. For negative staining, the samples were placed on a carbon-coated grid and stained with 1% uranyl acetate. Thin-section samples were prepared by N. Ghori, Department of Microbiology and Immunology, Stanford University.

Surface attachment assay. Cells (2 μ l) cultured overnight in LB were inoculated into 100 μ l of LB in 96-well polyvinyl chloride plates (Falcon 3911 Microtiter III flexible assay plate; Becton Dickinson, Oxnard, Calif.) and incubated at 30°C without shaking for 24 h. After the medium was removed, each well was rinsed with sterile water, 100 μ l of 1% crystal violet solution was added, and the plate was incubated for 15 min at room temperature. The wells were thoroughly washed with water and air dried. The adherent crystal violet was extracted with 200 μ l of 95% ethanol, diluted in 95% ethanol, and measured at 595 nm in a microplate spectrophotometer (model 550; Bio-Rad Laboratories, Hercules, Calif.).

P_i uptake assay. Cells were grown in a MOPS medium with 0.1 mM P_i overnight, collected, washed with a P_i -free MOPS medium, and resuspended in 50 ml of the P_i -free medium to an optical density at 600 nm (OD_{600}) of 0.1. The cultures were shaken for 2 h at 37°C and were readjusted to an OD_{600} of 0.1 by dilution with the P_i -free MOPS medium. K_2HPO_4 (0.1 mM) with [^{32}P] P_i (1 μ Ci/ml) was added to the shaking cultures, and samples (0.1 ml) were taken at intervals and immediately filtered through an HA filter (3.5-mm diameter; Millipore). The cells trapped on the filter were washed with 10 ml of P_i -free MOPS medium without glucose, amino acids, or vitamins. The radioactivity on the membrane filter was measured in a liquid scintillation counter; the values of P_i uptake in the cells are indicated as counts per minute per milliliter per OD_{600} unit.

Purification of *V. cholerae* PPK. *E. coli* strain CF5802 transformed with pVCK31 was used as the starting material (4.2×10^8 U in 610 mg of protein). The purification was performed essentially as described previously (2), modified only by the addition of a phenyl Sepharose column (Amersham Pharmacia Biotech, Inc.) as the final step (yielding 4.6×10^6 U in 0.12 mg of protein).

Nucleotide sequence accession number. The GenBank accession number for the sequence reported here is AF083928.

RESULTS

Poly P accumulation. When *E. coli* was grown in rich medium (LB) under normal conditions (37°C with aeration), poly P levels did not exceed 1 nmol (in P_i residues) per mg of total cell protein at any stage (21). The highest levels of transient poly P accumulation, about 25 nmol/mg of protein, occur when *E. coli* cells are exposed to specific stresses (4). However, *V. cholerae* strain 92A1552-Rif^r, even when grown in LB, accumulated poly P to levels of more than 50 nmol/mg (Fig. 2A) during logarithmic growth, while in stationary-phase cells the levels were low (~ 1 nmol/mg). In MOPS minimal salts medium, *V. cholerae* accumulates more than 20 nmol of poly P/mg in stationary phase, far exceeding the <1 -nmol/mg levels for *E. coli* (21). Thus, *V. cholerae* accumulates significantly higher levels of poly P than *E. coli* at all stages: during growth in rich media and during stationary phase in minimal media.

To determine whether the high level of poly P accumulation in *V. cholerae* relative to that in *E. coli* is due to a higher level of PPK, we measured PPK activity in *V. cholerae* and found that the membrane fraction activity (Table 1) was quite similar to that of *E. coli* MG1655. PPK activity levels were virtually the same in logarithmic- and stationary-phase cells (data not shown). Thus, poly P accumulation levels do not reflect the levels of PPK activity, which is also true in *E. coli* (21). In contrast to *E. coli*, no detectable activity of PPX was observed in *V. cholerae* (Table 1). Since a measurable level of PPX activity was detected in an *E. coli* $\Delta ppk \Delta ppx$ strain bearing a plasmid with the *V. cholerae ppk ppx* operon (see below), *V. cholerae* PPX should be expressed. The undetectable level of native PPX activity may explain in part why *V. cholerae* accumulates large amounts of poly P. The rapid degradation of poly

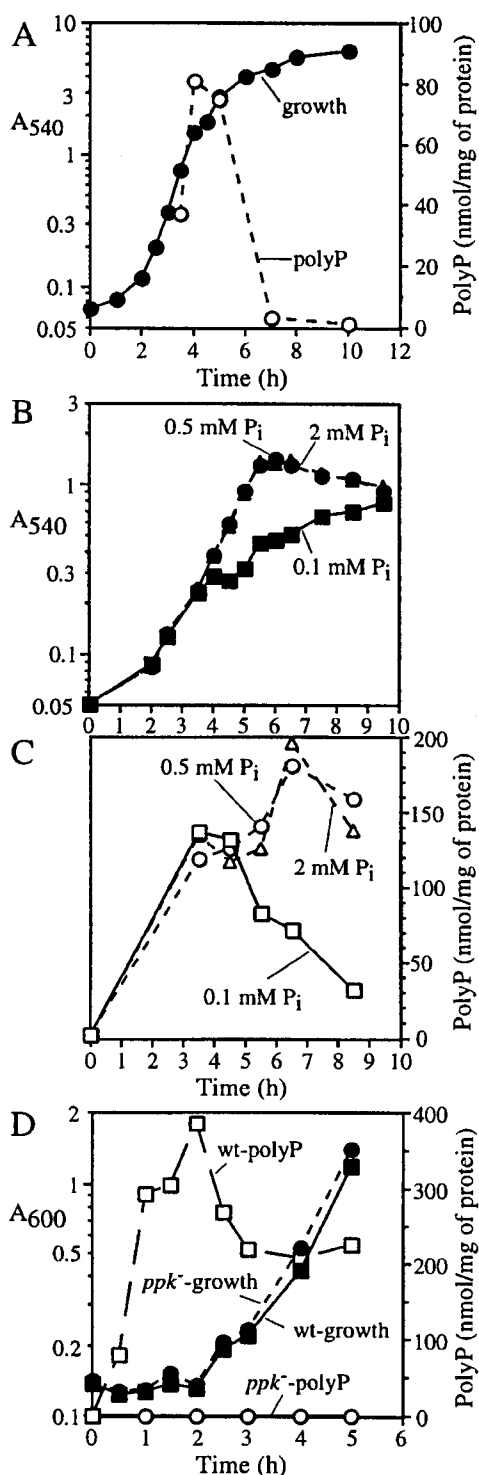


FIG. 2. Poly P accumulations in *V. cholerae*. (A) Following overnight culture in LB, the wild-type strain 92A1552-Rif^r was subcultured into fresh LB at a 1:100 dilution and grown at 37°C with aeration. (B and C) Following overnight culture in MOPS medium with 0.1 mM P_i, the wild-type strain was subcultured in MOPS medium with 0.1 (squares), 0.5 (circles), or 2 (triangles) mM P_i. Solid symbols, growth (A₅₄₀); open symbols, poly P. (D) Following overnight culture in MOPS with 0.1 mM P_i, the wild-type (squares) and KVC3 (*ppk* mutant [circles]) strains were harvested and washed with MOPS buffer. The cells were transferred to P_i-free MOPS and cultivated for 1 h at 37°C with aeration. Potassium phosphate buffer (pH 7.4) was then added to a final concentration of 20 mM P_i at zero hour. Solid symbols, growth (A₆₀₀); open symbols, poly P.

TABLE 1. PPK and PPX activities and poly P levels in *V. cholerae* and *E. coli*^a

Strain	Genotype ^b	PPK activity (U/mg)		PPX activity (U/mg)	Poly P level (nmol/mg)
		Soluble	Membrane		
<i>V. cholerae</i>					
92A1552-Rif ^r	WT	1,120	17,300	<30	25.3
KVC3	<i>ppk</i> ::Kan	<30	<300	<30	<0.10
<i>E. coli</i> MG1655					
	WT	550	14,000	1,200	

^a PPK and PPX activities were determined using cell extracts prepared from stationary-phase cultures in LB, and poly P levels were determined using cell extracts prepared from stationary-phase cultures in 2 mM P_i MOPS medium.

^b WT, wild type.

P shown in Fig. 1A, in the absence of significant PPX activity, may be due to the reverse reaction of PPK (27), in which poly P is converted to ATP. A *Klebsiella aerogenes ppk* mutant strain demonstrated profiles for poly P accumulation and degradation similar to those of the parental wild-type strain (A. Kuroda, personal communication).

Poly P overplus. The influence of P_i concentration on poly P accumulation was determined in cells grown in a defined medium (MOPS) (Fig. 2B and C). Poly P levels were below the detection limit (<0.5 nmol/mg) when cultivated with 0.1 mM P_i overnight (Fig. 2C, 0 h). However, >100 nmol/mg accumulated after 4 h in log phase with 0.1, 0.5, or 2 mM P_i. Cultures with 0.5 or 2 mM P_i maintained poly P levels of >100 nmol/mg for up to 9 h, i.e., after entering stationary phase. However, poly P levels in the 0.1 mM P_i culture decreased gradually along with the decrease in growth rate (Fig. 2B and C, 5 to 9 h). Given the similarity in the growth curves for the 0.5 and 2 mM P_i cultures, the slow growth of the 0.1 mM P_i culture is likely due to P_i depletion. Thus, poly P levels in *V. cholerae* depend on the P_i concentration in the medium.

When cells were shifted from a MOPS medium with no P_i added to the same medium with 20 mM P_i, dramatic accumulations of poly P occurred immediately after the upshift (Fig. 2D). Within 1 h, poly P accumulated to >300 nmol/mg and to near 400 nmol/mg after 2 h without any concomitant growth. With the resumption of growth, the poly P level decreased to 250 nmol/mg and remained there for at least five more hours. Similarly, large poly P accumulations have been reported in *Aerobacter aerogenes* as the "poly P overplus" phenomenon (9).

These massive levels of poly P were observed by electron microscopy as numerous bodies of relatively high electron density (Fig. 3). About 1 in 30 cells contained bodies 20 to 40 nm in diameter (Fig. 3B), while the others had smaller bodies, about 5 nm in diameter (Fig. 3A). Such granules were not found in wild-type cells grown in P_i-free medium or in *ppk* mutant cells (see below) grown in P_i-rich medium for 2 h (Fig. 3C and D). Inasmuch as these bodies were correlated with the massive accumulations of poly P, they are presumed to be poly P. Similar granules observed in *Myxococcus xanthus* (9) and *Helicobacter pylori* are somewhat localized at the flagellar pole and in association with the inner membrane as well as being dispersed in the cytoplasm (5). As determined by thin-section electron microscopy (Fig. 3E), the granules in *V. cholerae* are distributed in the cytoplasm but not localized along the inner membrane.

The *ppk* gene. The region in the TIGR *V. cholerae* genome database surrounding a sequence homologous to that of *E. coli ppk* was cloned from a *V. cholerae* genome library by colony hybridization using a PCR fragment of the region as a probe (see Materials and Methods). The *E. coli Δppk Δppx* knockout

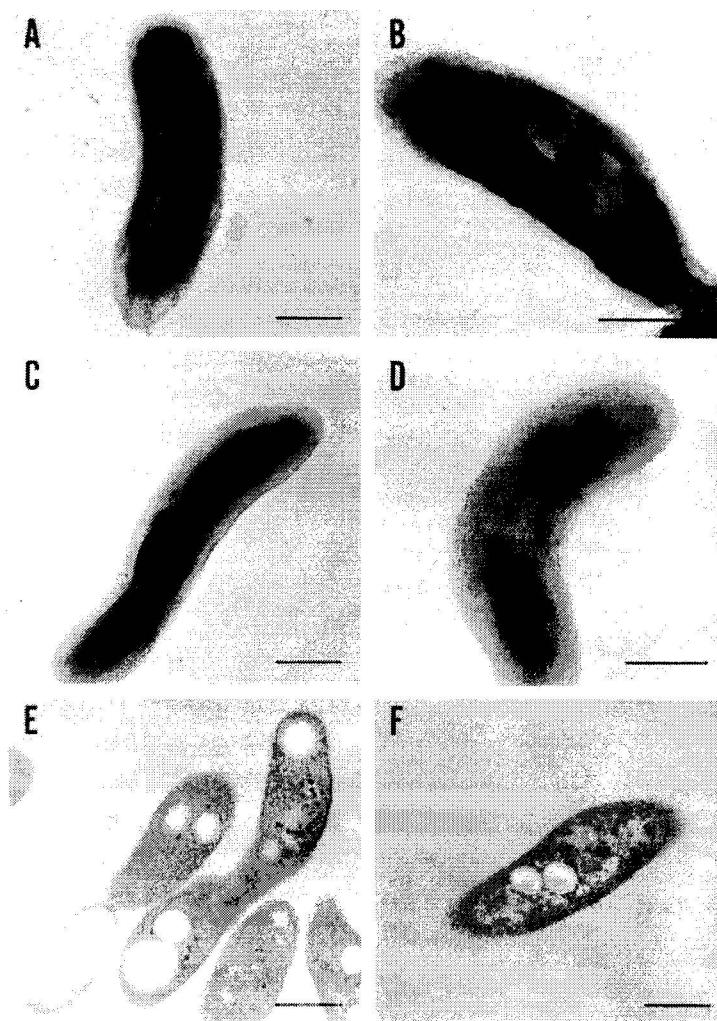


FIG. 3. Electron micrographs of *V. cholerae* strains. The bacteria were harvested at 0 and 2 h after the addition of P_i , as for Fig. 2D. Bars, 0.5 μm . Negatively stained samples were prepared from the wild-type culture after 0 (C) and 2 (A and B) h of incubation and from KVC3 (the *ppk* mutant) after 2 h of incubation (D). Thin-section samples were prepared from the wild type (E) and KVC3 (F) after 2 h of incubation. Magnifications, $\times 28,000$ (A, C, E, and F), $\times 35,000$ (D), and $\times 45,000$ (B).

strain CF5802, transformed with pVCK35 containing the *V. cholerae ppk* homolog (Fig. 1A), exhibited high levels of PPK activity (38,000 U/mg), demonstrating that this region did in fact contain the *V. cholerae ppk* gene; this was confirmed subsequently by sequencing. The deduced amino acid sequence of *V. cholerae* PPK is 701 amino acid residues long, with a calculated molecular mass of 81.6 kDa; it is 64% identical and 83% similar (in conserved residues) to that of *E. coli*. Sequencing also revealed a *ppx* homolog downstream of *ppk* in *V. cholerae*, as in *E. coli*, although with a two-cistron overlap. A transformant of the *E. coli* $\Delta ppk \Delta ppx$ mutant strain CF5802 harboring pVCK31, which contains both the *ppk* and *ppx* homologs (Fig. 1A), gave low but significant levels of PPX activity (540 U/mg). Its deduced amino acid sequence is 500 amino acid residues in length, with a calculated molecular mass of 56.4 kDa, and is 51% identical and 70% similar (in conserved residues) to *E. coli* PPX.

Upstream of the *ppk* open reading frame (ORF) (Fig. 1B) lies a putative promoter region which contains a probable *pho* box sequence with 15 of the 18 consensus base pairs at positions -84 to -67 from the A in the *ppk* start codon (Fig. 1B).

In *E. coli*, *pho* boxes are the binding sites of the two-component system regulator protein PhoB. Homologs of *phoB* and *phoR* (the cognate sensor) are found in the *V. cholerae* genome database, suggesting that transcription of the *ppk ppk* operon may be regulated by this system. Putative *pho* boxes are also present in the *ppk* promoter regions of *E. coli*, *K. aerogenes* (12), and *Acinetobacter* sp. strain ADP1 (8). A pair of 17-bp inverted-repeat sequences (from positions 24 to 40 and 43 to 59 from the *ppx* stop codon) appear to constitute a rho-independent transcriptional terminator site downstream of the *ppx* ORF (Fig. 1D). These features imply that *ppk* and *ppx* form an operon, as in *E. coli* and other gram-negative bacteria. The *E. coli* $\Delta ppk \Delta ppx$ transformant with a high copy number of the *V. cholerae ppk ppk* operon [CF5802(pVCK31)] expressed 690,000 U of PPK and 540 U of PPX activity per mg. We have shown previously that the *E. coli* wild type transformed with a high copy number of the *E. coli ppk ppk* operon exhibited 630,000 U of PPK and 50,000 U of PPX activity per mg (2, 3). Thus, *V. cholerae* PPK was expressed in an *E. coli* host cell at levels similar to those of *E. coli* PPK from the *E. coli* operon, but *V. cholerae* PPX was expressed about 100-fold less than *E.*

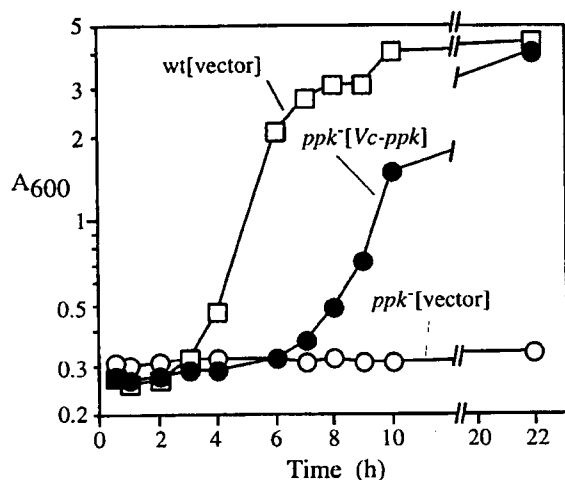


FIG. 4. *V. cholerae* *ppk* complements the adaptive growth defect in an *E. coli* *ppk* mutant. *E. coli* strains MG1655(pFYZ1) (squares), CF5802(pFYZ1) (open circles), and CF5802(pVCK37) (solid circles) were grown in 2×YT medium supplemented with 50 μg of ampicillin/ml to an A_{600} of 0.5. The cells were harvested and washed twice with MOPS medium devoid of nutrients and resuspended in MOPS medium with 2 mM P_i and 0.4% glucose at zero hour.

coli PPX. Unlike in *E. coli*, there is a 20-bp overlap between the *ppk* and *ppx* ORFs in the *V. cholerae* operon (Fig. 1C), which may interfere with translation of the *ppx* ORF from the *ppk-ppx* mRNA, accounting for the undetectable level of PPX activity (Table 1).

Null mutant of *ppk*. In a *ppk* knockout mutant in *V. cholerae* (KVC3) (see Materials and Methods), the levels of PPX activity and poly P accumulation were undetectable (Table 1 and Fig. 2D), in contrast to the parental strain, 92A1552-Rif^r, which accumulated more than 300 nmol of poly P per mg when shifted from a P_i -free to a 20 mM P_i defined medium. We tested KVC3 for the reported phenotypes of the *E. coli* *ppk* mutant strain CA10 (5, 18, 20). No phenotypes were observed for long-term survival in synthetic medium (for 30 days at 30°C), sensitivity to heat (at 45 and 55°C) and hydrogen peroxide (in 10, 3, and 1 mM), adaptive growth following a shift from rich medium to minimal medium, or long-term (10 days at 30°C) survival in artificial seawater (29).

With regard to adaptive growth in a nutrient downshift (Fig. 4), the *E. coli* wild-type strain MG1655 transformed with the plasmid vector (pFYZ1) grew in a minimal medium after a 2-h lag following downshift from a rich medium (LB) whereas the *E. coli* $\Delta ppk \Delta ppx$ mutant CF5802 harboring pFYZ1 was unable to grow even 22 h after the downshift. However, CF5802 bearing the *V. cholerae* *ppk* plasmid pVCK37 grew in minimal medium after the downshift with only a 4-h lag, after which the growth rate and final OD were similar to that of the wild type. Thus, the *V. cholerae* *ppk* complements the *E. coli* Δppk mutation in response to this stress condition.

A. P. aeruginosa PAO1 *ppk* mutant shows no defects in adaptive responses but is severely impaired in motility and surface attachment (22, 23, 24), and the *V. cholerae* *ppk* mutant was found to be deficient in these features as well (23). The swim area of the *ppk* mutant was 57% that of the wild type on 0.3% agar plates (Table 2). The *ppk* mutant also exhibited a significantly lower ability for surface attachment (Table 2). Deficiencies in motility and surface attachment have also been observed in *ppk* mutants of *E. coli*, *K. pneumoniae*, and *Salmonella* spp. (22).

TABLE 2. Swimming and surface attachment

Strain	Genotype	Swim area (cm ²) ^a	Surface attachment (A_{595}) ^b
92A1552-Rif ^r	WT ^c	1.52 ± 0.07	2.14 ± 0.70
KVC3	<i>ppk::Kan</i>	0.87 ± 0.07	1.46 ± 0.30

^a Data are from Rashid et al. (23).

^b Average of 16 measurements; two-tail *P* value by *t* test with a 0.05 threshold is 0.0012.

^c WT, wild type.

In view of the capacity of poly P to function as a chelator of divalent metals (9), the calcium sensitivity of the *V. cholerae* *ppk* mutant was tested (Fig. 5). The growth lag time of the wild-type strain (92A1552-Rif^r) in LB containing 200 mM CaCl₂ was 5 h, more than 4 h longer than in the absence of CaCl₂ (Fig. 2A). The lag time of the *ppk* mutant KVC3 was significantly longer, at 7 h. When complemented with the high-copy-number plasmid pVCK31 harboring the *ppk* gene, the lag time was shortened to 3 h. As both the wild type and the *ppk* mutant of *V. cholerae* had the same lag time when grown in LB in the presence of 400 mM NaCl (data not shown), these results imply that the *ppk* gene is involved in an adaptation to excess levels of calcium but not chloride or osmolality.

Another phenotype of the *ppk* mutant was observed with regard to P_i uptake (Fig. 6). After growth in a P_i -free medium for 2 h, wild-type cells displayed a linear (nonsaturable) P_i uptake for up to 25 min when incubated in 0.1 mM P_i (the P_i -limited condition) (Fig. 2B and C). The Δppk mutant had unique saturable profiles for P_i uptake. It showed a rate of uptake similar to that of the wild type from 0 to 3 min, but the uptake after 5 min was minimal and the rate was decreased significantly. These data suggest that *ppk* is required for the continual high-rate uptake of P_i under low- P_i conditions.

Characterization of PPX. PPX was purified from a transformant of the *E. coli* CF5802 strain ($\Delta ppk \Delta ppx::Kan$) bearing the *V. cholerae* *ppk ppx* operon on a high-copy-number vector, pVCK31. Homogeneity of the purified protein was verified as

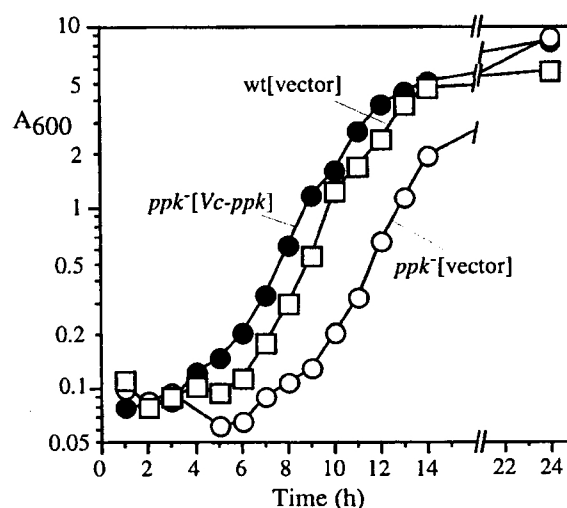


FIG. 5. Growth adaptation to an excess amount of calcium. *V. cholerae* strains 92A1552-Rif^r(pBluescript II) (squares), KVC3(pBluescript II) (open circles), and KVC3(pVCK31) (solid circles) were grown in LB medium supplemented with 10 mM KH₂PO₄ and 50 μg of ampicillin/ml overnight. The cells were inoculated in LB supplemented with 200 mM CaCl₂ and shaken at 37°C.

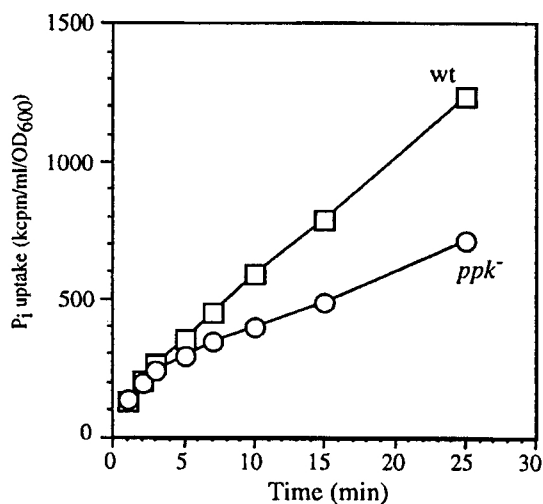


FIG. 6. P_i uptake of *V. cholerae*. P_i uptake activities of 92A1552-RiF (squares) and KVC3 (circles) were tested as described in Materials and Methods.

a single band on a Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel (data not shown) with a molecular mass estimated at 87 kDa, compared to the calculated mass of 81.6 kDa. Comparison of the PPK retention time to those of reference proteins in a high-performance liquid chromatography gel filtration column showed a molecular mass of 310 kDa (data not shown), indicating that *V. cholerae* PPK is a homotetramer like *E. coli* PPK (1). The final fraction has a specific activity of 40×10^6 U/mg, slightly higher than that of *E. coli* PPK (29×10^6 U/mg) (1).

The optimal reaction conditions for *V. cholerae* PPK are almost the same as those for *E. coli* PPK, e.g., a pH optimum of 7.2 in HEPES buffer and 4 mM Mg^{2+} , and 40 mM ammonium sulfate increases activity twofold. Autophosphorylated PPK was observed in a reaction with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Like the *E. coli* enzyme (16), the *V. cholerae* enzyme can catalyze the synthesis of ATP from ADP and poly P and can catalyze the synthesis of GTP and ppppG (linear guanosine 5'-tetraphosphate) from GDP and poly P. Significant differences in the kinetic parameters for the PPKs of *V. cholerae* and *E. coli* are shown by the 40-fold decrease in the *V. cholerae* K_m value for ATP for the forward (poly P synthesis) reaction (Table 3). The k_{cat}/K_m ratio for the ATP synthesis reaction of *V. cholerae* PPK is more than 10 times higher than that for *E. coli*. On the other hand, the k_{cat}/K_m s ratios for the GTP and ppppG synthesis reactions of *V. cholerae* PPK are 5 and 20 times lower, respectively, than those for *E. coli*. These data suggest that the *V. cholerae* PPK is more specific for the generation of ATP than GTP or ppppG.

DISCUSSION

Accumulations of poly P in *V. cholerae* are remarkable for being so great and sustained compared to those in *E. coli*. The levels in excess of 50 nmol/mg of protein during exponential growth in a rich medium (Fig. 2A) and 150 nmol/mg in stationary phase in a defined medium (Fig. 2C) are roughly 100 times those in *E. coli*. Yet the PPK activities in extracts are nearly the same (Table 1). Some of the reason may lie in the undetectable levels of PPK activity in extracts of *V. cholerae* (Table 1). In that organism, as in *E. coli* (3), an operon contains the *ppk* as well as the *ppx* gene (Fig. 1). Unlike in *E. coli*,

where the *ppx* gene is separated by 7 bp from the upstream *ppk* gene, the amino terminus-encoding region of *ppx* in *V. cholerae* overlaps the carboxy terminus-encoding region of *ppk* by 20 bp (Fig. 1C). The *E. coli* transformant with the *V. cholerae* *ppx* gene on a high-copy-number plasmid did express PPK activity. How these genes and possibly others are regulated pre- and posttranscriptionally remains to be determined.

When cells are switched from a P_i starvation medium to one with adequate P_i , there is a massive accumulation of poly P (Fig. 2D), as observed in *A. aerogenes* (9) and designated the poly P overplus phenomenon. The accumulation of poly P is evident as electron-dense granules up to 40 nm in diameter with an ordered matrix structure as in crystal complexes (Fig. 3B). The granules appear to be largely cytoplasmic (Fig. 3E), unlike some of the granules in *H. pylori*, which have polar and membrane-oriented locations (5). The dynamic accumulation and removal of poly P and its mobilization at a molecular level, as well as the nature of the granules and their cellular locations, need to be clarified.

V. cholerae PPK resembles that of *E. coli* in size and in its multiple activities: processive poly P synthesis from ATP, nucleoside diphosphate kinase action on ADP and GDP by donor poly P, pyrophosphoryl transfer to GDP to form ppppG, and autophosphorylation by ATP. The most notable differences are in the kinetic parameters (Table 3), e.g., a K_m for ATP of 0.2 mM for *V. cholerae* PPK compared to 2.0 mM for *E. coli* PPK. Also, kinetic parameters for ATP, GTP, and ppppG synthesis reactions indicate that *V. cholerae* PPK is more specific for the generation of ATP than for GTP or ppppG compared to *E. coli* PPK. These enzyme characteristics are similar to those of *H. pylori* PPK purified as a recombinant protein (27; C.-M. Tzeng and A. Kornberg, unpublished data).

The strong PPK sequence homologies of 20 or more bacterial species include a number of the major pathogens (26), *V. cholerae* among them. In view of the striking dependence of *E. coli* on PPK for a variety of adaptive responses in the stationary phase and the expression of virulence factors in the stationary phases of some pathogens (7), the phenotypes of null mutants of *ppk* in these pathogens have been sought. Furthermore, there is the attractive possibility that PPK might prove a novel target for an antimicrobial drug with a broad spectrum and minimal side effects, inasmuch as PPK has not been found in animal species.

Among the pathogenic features of the PPK null mutants, a decrease in motility (commonly associated with a loss of virulence [19]) and weakened attachment to abiotic surfaces (a

TABLE 3. Kinetic parameters of PPK

Reaction and source	K_m (mM)	V_{max} (pmol mg^{-1} min^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Poly P synthesis				
<i>V. cholerae</i>	0.05	1.6×10^6	8.2	164
<i>E. coli</i> ^a	2	51×10^6	59	29.5
ATP synthesis				
<i>V. cholerae</i>	0.12 ^b	95×10^6	65.3	544
<i>E. coli</i> ^c	0.25	3.7×10^6	10.5	42
GTP synthesis				
<i>V. cholerae</i>	2.5 ^b	1.5×10^4	0.076	0.03
<i>E. coli</i> ^c	0.63	5.7×10^4	0.088	0.14
ppppG synthesis				
<i>V. cholerae</i>	2.3 ^b	1.1×10^3	0.006	0.002
<i>E. coli</i>	0.46	9.0×10^3	0.027	0.04

^a *E. coli* data for poly P synthesis are from Ahn and Kornberg (1).

^b Values for ADP or GDP.

^c *E. coli* data for ATP and GTP synthesis are from Kuroda and Kornberg (16).

frequent correlate of poor biofilm formation) have been observed in several pathogens. Particularly striking are mutants of *P. aeruginosa* (22, 23, 24) which are defective in quorum sensing and have also lost their virulence in mouse models. The *V. cholerae ppk* mutant also had diminished attachment to an abiotic surface (Table 2), with the activity decreased by 68% compared to that of the wild type. Although this was not a dramatic reduction as with *P. aeruginosa* (22, 24), it could be emphasized by using the rugose colony variant of *V. cholerae* O1 (30), which prefers to form biofilm, compared to the smooth colony variant which was used in this study.

The failure to adapt to stress and the lack of survival in stationary phase observed in the *E. coli ppk* mutant (6) have not been apparent in the *V. cholerae* mutant. In addition to decreased motility and decreased attachment to abiotic surfaces (Table 2), other defects have been observed. One is a delayed adaptation to high calcium levels in the medium (Fig. 5); complementation of the mutant with the *ppk* gene more than corrects for the extended lag in growth. It is plausible that a large amount of poly P can trap excess calcium in the cell and maintain the calcium level at a low enough level to permit growth. Another defect of the *ppk* mutant is an abnormal rate of uptake of P_i from the medium (Fig. 6). Whereas the initial rate resembled that of the wild type, the subsequent rate was considerably reduced. Not enough is known about P_i uptake in *V. cholerae* to identify which system might be affected by the lack of PPK function. Interestingly, budding-yeast mutants deficient in poly P accumulation also showed a similar saturable curve for P_i uptake (18a).

The phenotypic tests of the *V. cholerae ppk* mutant were patterned on those performed on bacterial species which differ sharply from *V. cholerae* in their physiologic features, commensal interactions, and host invasiveness (10). In view of the unique aspects of the aquatic ecology of *V. cholerae*, much needs to be studied to evaluate what effect the lack of PPK and poly P might have on its survival and pathogenesis.

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