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Vibrio cholerae phosphatases required for the utilization of nucleotides and extracellular DNA as phosphate sources

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Summary

Phosphate is essential for life, being used in many core processes such as signal transduction and synthesis of nucleic acids. The waterborne agent of cholera, *Vibrio cholerae*, encounters phosphate limitation in both the aquatic environment and human intestinal tract. This bacterium can utilize extracellular DNA (eDNA) as a phosphate source, a phenotype dependent on secreted endo- and exonucleases. However, no transporter of nucleotides has been identified in *V. cholerae*, suggesting that in order for the organism to utilize the DNA as a phosphate source, it must first separate the phosphate and nucleoside groups before transporting phosphate into the cell. In this study, we investigated the factors required for assimilation of phosphate from eDNA. We identified PhoX, and the previously unknown proteins UshA and CpdB as the major phosphatases that allow phosphate acquisition from eDNA and nucleotides. We demonstrated separable, but partially overlapping roles for the three phosphatases and showed that the activity of PhoX and CpdB is induced by phosphate limitation. Thus, this study provides mechanistic insight into how *V. cholerae* can acquire phosphate from extracellular DNA, which is likely to be an important phosphate source in the environment and during infection.

Keywords

Vibrio cholerae; phosphatases; nucleotidases; eDNA

Introduction

As a waterborne, facultative pathogen, *V. cholerae* transits between the aquatic environment and the human intestinal tract. During life in the environment, *V. cholerae* is often found in biofilms associated with phyto- and zooplankton but may also persist in a planktonic state (Lipp, *et al.* 2002). The bacteria enter the human host via ingestion of contaminated water or food and subsequently colonize the small intestine (Peterson 2002). Expression of cholera toxin, an ADP-ribosylating enterotoxin, leads to excessive release of water from intestinal epithelial cells, resulting in massive secretory diarrhea and rapid dehydration of the host (Childers and Klose 2007; Faruque 2011; Herrington *et al.*, 1988; Peterson 2002). Expulsion from the host typically results in delivery of the bacteria back into aquatic reservoirs.

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V. cholerae encounters phosphate limitation both in the aquatic environment and within the human host (Kamal *et al.*, 2007; McDonough *et al.*, 2014; Nelson *et al.*, 2008; Schild *et al.*, 2007). Due to the importance of phosphate, bacteria, including *V. cholerae*, have evolved several mechanisms to acquire it from the environment. Inorganic phosphate is the most readily available form of phosphate in the aquatic environment (White and Metcalf 2007) and most bacteria encode two independent systems for its uptake into the cytoplasm: inorganic phosphate transport system (PitA or PitB) and phosphate-specific transport system (Pst/PhoU). *V. cholerae* harbors a functional Pst/PhoU system (Heidelberg *et al.*, 2000; McDonough *et al.*, 2014; Pratt *et al.*, 2009), as well as an uncharacterized gene (VC2442) that encodes a protein with homology (37% identity) to the *E. coli* PitA.

Some bacteria are able to utilize organophosphates, which are characterized by a phosphorous-oxygen-carbon ester bond (*e.g.* sugar phosphates), as sources of phosphate. Although many organophosphates can cross the outer membrane of Gram-negative bacteria, they cannot be transported into the cytoplasm of cells with the exceptions of glycerol-3-phosphate and hexose-6-phosphates (van Veen 1997, Lamarche, *et al.* 2008). Extra-cytoplasmic phosphatases can facilitate the removal of phosphate groups from non-transportable phosphate-compounds. For example, *V. cholerae* alkaline phosphatase (PhoX), which is expressed in the periplasm, is able to remove the phosphate group from several organic phosphate compounds including glucose-6-phosphate, glucose-1-phosphate, and β-glycerophosphate (Roy, *et al.* 1982).

Extracellular DNA (eDNA), which represents a major class of organophosphate compound, is present in picomolar to micromolar concentrations in aquatic environments, depending on the location tested (Bjorkman and Karl 2005; Lorenz and Wackernagel 1994). The source of the DNA in aquatic environments is unclear, although much of it may be released from decomposing microbes, zooplankton, fish, or other aquatic-dwelling organisms. Additionally, several marine- and fresh water-dwelling bacterial species were found to secrete DNA under exponential growth conditions (Nielsen *et al.*, 2007; Paul and David 1989). The purpose of DNA secretion in microbes has been tied with biofilm formation, as eDNA can help provide structure to a biofilm matrix (Qin *et al.*, 2007; Whitchurch *et al.*, 2002). However, several organisms, including *Ruegeria pomeroyi*, *Yersinia entericolitica*, *Pseudomonas aeruginosa*, *Shewanella* spp. and *Corynebacterium glutamicum*, have been shown to utilize DNA and/or nucleotides as sources of phosphate, carbon, and nitrogen (Mulcahy *et al.*, 2010; Pinchuk *et al.*, 2008; Rittmann *et al.*, 2005; Sebastian and Ammerman 2011; Trulzsch *et al.*, 2001).

Seper and coworkers (2011) recently demonstrated that *V. cholerae* is also able to utilize eDNA as a sole source of phosphate. During its life cycle, *V. cholerae* may encounter eDNA in both the aquatic environment or in the host. The aquatic environment provides ambient eDNA (as discussed above), but eDNA is also found within the matrix of *V. cholerae* biofilms (Seper *et al.*, 2011). Within a host, *V. cholerae* can stimulate the release of neutrophil extracellular traps (NETs), which are web-like structures comprised of neutrophil secreted-eDNA and antimicrobial proteins (Branzk and Papayannopoulos 2013; Kawasaki and Iwamuro 2008; Zawrotniak and Rapala-Kozik 2013). Thus DNA from these NETs could potentially provide a source of nutrients for the organism (Seper *et al.*, 2013).

Utilization of eDNA as a source of phosphate requires the break down of eDNA into nucleotides. *V. cholerae* produces and secretes two nucleases into culture supernatant: Xds, with exonuclease activity; and Dns, with endonuclease activity (Focareta and Manning 1987; Focareta and Manning 1991a; Focareta and Manning 1991b; Seper *et al.*, 2011). These nucleases are both induced under low phosphate conditions, suggesting a role in phosphate scavenging during starvation conditions (McDonough *et al.*, 2014; Seper *et al.*, 2011). Although the main role of Xds and Dns may be to break down structural eDNA within a biofilm matrix or in NETs, it is not surprising that both are required for wild type growth on eDNA as a sole source of phosphate (Seper *et al.*, 2011; Seper *et al.*, 2013), since their activity presumably results in extracellular accumulation of nucleotides. In order to access the phosphate from these nucleotides, *V. cholerae* must separate the phosphate group from the nucleoside group through the action of one or more phosphatases.

In this work we aimed to identify the phosphatases required for release of phosphate from nucleotides (*i.e.* nucleotidases). Nucleosides, which lack a phosphate group, are readily transported into the cytoplasm of *V. cholerae* via Nup transporters (Gumpenberger, *et al.* [accompanying manuscript from separate group]). However, no nucleotide transporter has been identified, suggesting that the phosphorylated nucleosides remain in the periplasm (Watanabe *et al.*, 2011). Therefore, we hypothesized that expression of a periplasmic or extracellular phosphatase is required for growth on eDNA by releasing phosphate from the mononucleotides liberated by Xds and Dns. Presumably, once the phosphate is removed, it can be taken up into the cell by any of the phosphate transporters, *i.e.* Pst/PhoU or Pit. Here, we have presented evidence that three phosphatases, PhoX, UshA, and CpdB are the major phosphatases contributing to the ability of *V. cholerae* to assimilate phosphate from eDNA.

Results

PhoX is not required for utilization of eDNA as a phosphate source

Several organisms including *R. pomeroyi* and *Shewanella* spp., use alkaline phosphatase to remove phosphate from nucleotides (Pinchuk *et al.*, 2008; Sebastian and Ammerman 2009). The *V. cholerae* alkaline phosphatase, PhoX, is expressed in the periplasm and the gene is regulated by the major phosphate starvation response regulator, PhoB (Pratt *et al.*, 2009; von Kruger *et al.*, 2006). Therefore, we hypothesized that PhoX would provide the required phosphatase activity for growth on eDNA as a source of phosphate for *V. cholerae*.

To test our hypothesis, we assessed the growth of wild type and Δ*phoX* strains in MOPS-glucose minimal medium that was either lacking phosphate or supplemented with sheared salmon sperm DNA as the sole source of phosphate (Figure S1). Prior to growth in the assay medium, the strains were pre-grown in MOPS-glucose minimal medium under phosphate replete conditions and washed thoroughly in the medium lacking phosphate. In this experiment, and in all other growth curves presented in this paper, strains were grown in no phosphate MOPS-glucose medium as a negative control. Additionally, all strains were grown in MOPS-glucose medium supplemented with 10 mM KH_2PO_4 (high phosphate), to ensure that the strains were able to grow equally well in phosphate replete conditions (data not shown). We included the Δ*xds*Δ*dns* strain, which is unable to utilize eDNA as a source of phosphate (Seper *et al.*, 2011), as a negative control to ensure that there was no

contaminating phosphate in the salmon sperm DNA. As had been reported earlier, we found that wild type *V. cholerae* was able to use eDNA as a source of phosphate, however the rate of growth was severely decreased when compared to wild type growth even under phosphate limiting – 0.1 mM KH_2PO_4 – conditions. Growth of the *phoX* deletion strain in eDNA closely matched the wild type. Thus, we concluded that either *phoX* does not contribute to utilization of eDNA as a source of phosphate, or its role is redundant with other phosphatases/nucleotidases.

Of note, wild type, Δ*phoX*, and Δ*xds*Δ*dns* all doubled a few times in medium lacking any phosphate source. This suggested that these strains accumulate internal phosphate stores (*e.g.* poly-phosphate) during one of the pre-growth conditions, and resort to utilizing this phosphate store upon transition into phosphate limiting environments. In this experiment and in all other eDNA growth curves, we saw day-to-day variation in growth rate of the strains, but very little variation between biological replicates within a single experiment. We believe this variation between experiments is connected to differences in phosphate storage of the bacteria prior to growth in the test media. Due to the growth rate variability, we did not calculate growth rates for the eDNA curves, and we have plotted only replicates from a single experiment.

Identification of additional putative phosphatases

We performed a genetic screen in order to identify additional phosphatases that may contribute to growth of *V. cholerae* on eDNA. A Δ*phoX* strain was mutagenized with a mTn*10* transposon and plated on LB plates containing the colorimetric phosphatase substrate, 5-bromo-4-chloro-3-indolyl phosphate (XP), which turns blue upon removal of the phosphate group and subsequent oxidation of the molecule. Colonies exhibiting phosphatase activity (*e.g.* wild type and Δ*phoX*) are blue on XP plates due to product accumulation in the periplasm, whereas colonies lacking phosphatase activity are white. Approximately 40,000 mTn*10* mutants were screened on LB XP plates and 7 white colonies were identified. Of these 7 colonies, 5 represented unique insertion sites present throughout the VC2174 coding region and 2 unique insertions were in the 5’ end of VC2352 (Figure S2). An in-frame deletion of VC2174 in the wild type background results in white colonies on LB XP plates, validating the transposon screen results. However, a clean deletion of VC2352 was still blue on LB XP plates, even when the VC2352 deletion was moved into the Δ*phoX* strain. VC2352, encoding a NupC-homolog, appears to be the dominant nucleoside transporter in *V. cholerae* (Gumpenberger, *et al.* [accompanying manuscript from separate group]). Our work suggests that expression of a truncated VC2352 somehow inhibits transport and/or cleavage of XP; however, since we can see no obvious tie to phosphatase activity, we did not further characterize this protein.

VC2174 is annotated as a bifunctional UDP-sugar hydrolase/5’nucleotidase (Heidelberg *et al.*, 2000), called UshA in bacteria. The online program, PSORT (http://www.psort.org/psortb/index.html) categorizes the protein translation of VC2174 as periplasmic, consistent with it having a predicted secretion signal sequence according to SignalP (http://www.cbs.dtu.dk/services/SignalP-4.1/) (Nakai and Kanehisa 1991; Petersen *et al.*, 2011). UDP-sugar hydrolases are a broad class of enzymes with two catalytic activities; 1) UDP-

sugar to UMP and sugar-phosphate, and 2) UMP to uridine and phosphate (Neu 1967a). In general, UDP-sugar hydrolases are extra-cytoplasmic and have broad substrate specificity for deoxy- and ribo-nucleotides (Neu 1967b; Rittmann *et al.*, 2005). UshA is a highly conserved protein among bacterial species. The enzymatic function is most thoroughly studied in *E. coli*, but its physiological function remains unclear in this species. In *C. glutamicum* and *Shewanella* spp. UshA is essential for growth when eDNA and/or nucleotides is supplied as the sole source of phosphate (Pinchuk *et al.*, 2008; Rittmann *et al.*, 2005). BLAST analysis revealed that *V. cholerae* UshA has 79%, 41%, and 72% sequence identity to UshA of *E. coli*, *C. glutamicum*, and *Shewanella oneidensis*, respectively (Altschul *et al.*, 1997; Altschul *et al.*, 2005). Based on its identity to UshA in other organisms, as well as work presented in this manuscript, we have designated VC2174 as *ushA*.

Using the UshA sequence as a query for a BLAST analysis (Altschul *et al.*, 1997; Altschul *et al.*, 2005) of the *V. cholerae* genome and by searching the *V. cholerae* genome annotation for “nucleotidase” (Heidelberg *et al.*, 2000), we identified three additional putative extra-cytoplasmic nucleotidases: VCA0545, VCA0608, and VC2562 (Table S1). VCA0545 harbors a 5’nucleotidase domain and is 62% similar to UshA of *S. oneidensis*. This suggests that VCA0545 may represent an additional 5’nucleotidase present within the bacterium. VCA0608 does not share homology with traditional nucleotidase proteins. The gene encodes a protein carrying a haloacid dehalogenase-like hydrolase domain (HAD), and is annotated as a provisional dUMP phosphatase. VC2562 is in the class of 2’3’cyclic phosphodiesterases. These enzymes have two independent active sites that catalyze the two-step reaction: 1) 2’3’cyclic nucleotide to 3’ nucleotide, and 2) 3’nucleotide to nucleoside and phosphate (Anraku 1964a; Anraku 1964b). Although 2’3’cyclic phosphodiesterase and 3’nucleotidase activities have been described in several organisms including many of the Enterobacteriaceae, the physiological role of this enzyme is not well characterized (Neu 1968). CpdB – the most commonly studied 2’3’cyclic phosphodiesterase in bacteria – of *Yersinia pestis* and *Y. enterocolicita* is essential for growth on 2’3’cAMP as the sole source of carbon. VC2562 shares 81% and at least 80% identity to CpdB of *E. coli* and *Yersinia* spp., respectively (Altschul *et al.*, 1997; Altschul *et al.*, 2005). Based on this high level of identity to CpdB in other organisms, as well as work presented in this manuscript, we have designated VC2562 as *cpdB*.

In order to determine if UshA, CpdB, VCA0545, or VCA0608 contribute to growth of *V. cholerae* on eDNA, we made a quintuple knock-out strain (Δ5 phosphatases) in which all four putative nucleotidases were deleted in conjunction with *phoX*. We tested this strain for its ability to utilize eDNA as a source of phosphate as described for the *phoX* single mutant. We found that a strain which was deleted for all five putative phosphates was unable to grow using eDNA as a source of phosphate (Figure 1). Thus, we conclude that we have identified all major phosphatases involved in acquisition of phosphate from eDNA.

An unrelated finding in this experiment came from our using the Δ*xds*Δ*dns* as a negative control. Surprisingly, we found that this double mutant is able to reach wild type density, but with a much delayed growth rate. The growth of the Δ*xds*Δ*dns* strain is not evident until at least 50 hours into the incubation, which explains why Seper and coworkers (2011) did not

observe this phenotype. Presumably an additional, weak extracellular nuclease is present under this condition and contributes to the eDNA growth phenotype.

UshA is a non-nucleotide-specific 5'nucleotidase

The main product of eDNA degradation by Xds and Dns is thought to be monophosphorylated deoxynucleotides. We hypothesized that during growth on eDNA, the phosphate is removed from the mononucleotides, rather than the intact DNA strands. Therefore, in order to identify which of the five putative phosphatases are major contributors to the growth on eDNA, we performed growth curves in which 5'deoxy-mononucleotides were supplied as the sole source of phosphate (Figure 2 and Table 1). We identified that *ushA* is dominantly, and likely solely, responsible for supporting growth on all four 5'deoxy-mononucleotides; the growth of Δ*ushA* on all four nucleotides was comparable to that of the no phosphate control (Figure 2a–d and Table 1). The deletion of *ushA* was complemented *in trans* by expression of *ushA* from an IPTG-inducible promoter carried on a pMMB67EH vector (Figure 2e).

We performed nucleotidase assays, which use a mixture of ascorbic acid and molybdate to detect phosphate in solution upon release by nucleotidase activity, to continue assessment of UshA's 5'nucleotidase activity (Edwards *et al.*, 1993). Using whole cell lysates as a source of UshA protein, we expected that the wild type bacterium would harbor 5'nucleotidase activity, while the *ushA* mutant would have undetectable activity. Indeed, *V. cholerae* cell lysate harbored 5'nucleotidase activity against all four 5'deoxy-mononucleotides, and that activity was drastically reduced in the Δ*ushA* strain (Figure 3 and Table S2). We saw no detectable accumulation of phosphate in either a no substrate control or a substrate only control (data not shown).

Since *ushA* appeared to be the only phosphatase required for growth on 5'deoxy-mononucleotides, we hypothesized that it may account for the ability of *V. cholerae* to grow on eDNA as a source of phosphate. However, we found that the Δ*ushA* strain grew at a similar rate to the wild type strain under this condition (Figure 4). Thus, we concluded that while *ushA* accounts for all measurable 5'nucleotidase activity in the bacterium, at least one other phosphatase (*phoX*, *cpdB*, VCA0545, or VCA0608) also supports growth of *V. cholerae* on eDNA.

CpdB is a purine-specific 3'nucleotidase

Most DNases release 5'nucleotides, such that the phosphate group on the released nucleotide is attached to the 5' carbon. However, the precise activities of the *V. cholerae* Xds and Dns extracellular nucleases have not been demonstrated, and thus it is unclear what nucleotide-related substrates are produced by their activity on eDNA. BLAST analysis suggested that while Dns has strong identity to EndA-type endonucleases that release 5'nucleotides, Xds harbors a YhcR domain (Altschul *et al.*, 1997; Altschul *et al.*, 2005). The YhcR domain is named after the protein in which it was described, YhcR of *Bacillus subtilis* an endonuclease that releases 3'monophosphorylated nucleotides (Oussenko *et al.*, 2004; Seper *et al.*, 2011). Therefore, it seems likely that *V. cholerae* has the capacity to release both 5' and

3’phosphorylated mononucleotides. We hypothesized that the nucleotidase acting in concert with UshA during growth on eDNA is removing phosphate from 3’deoxy-mononucleotides.

The single phosphatase deletion strains were tested in growth assays in which 3’mononucleotides were supplied as the sole source of phosphate (Figure 5 and Table 1). Neither 3’dAMP or 3’dCMP were commercially available, so we used the ribo-nucleotide form of these molecules. First, we found that *V. cholerae* is unable to utilize 3’CMP as a source of phosphate (Table 1). Second, the only gene contributing to growth on 3’AMP, 3’dGMP, or 3’TMP appeared to be *cpdB* (Figure 5a,b,d), consistent with its being annotated as a 3’nucleotidase. While 3’TMP did support growth of *V. cholerae*, it was only slightly greater than the no phosphate control. Thus, we suggest that CpdB has a preference for purine nucleotides. We were able to restore *V. cholerae* growth on 3’dGMP by expressing *cpdB in trans* (Figure 5e).

In order to further support our finding that *cpdB* is a 3’nucleotidase, we performed nucleotidase assays using each of the four 3’mononucleotides as substrates. As expected from the growth assays, we found that wild type *V. cholerae* is able to remove the phosphate group from 3’AMP, 3’dGMP, and 3’TMP, but not from 3’CMP (Figure 6 and Table S3). However, while 3’dGMP supported full growth of wild type *V. cholerae*, the nucleotidase activity on this nucleotide is low, comparable to the activity on 3’TMP. This may be due to differences in how the bacteria were grown between the two assays (*e.g.* high phosphate LB for nucleotidase assays vs. phosphate limiting MOPS-glucose medium for the growth assays), and is addressed in Figure 9. As expected, the Δ*cpdB* strain exhibited no detectable nucleotidase activity on any of the 3’mononucleotides, supporting its classification as a 3’nucleotidase.

UshA, CpdB, and PhoX account for the majority of phosphatase activity required for growth on eDNA as a source of phosphate

Our work demonstrated that UshA and CpdB are required for growth on 5’ and 3’nucleotides as sole sources of phosphate, respectively. Therefore, we hypothesized that deletion of both genes would result in the loss of *V. cholerae* growth when eDNA is supplied as the sole source of phosphate. To test this, we performed a growth assay with wild type, Δ*ushA*Δ*cpdB*, and Δ*xds*Δ*dns*. While the double phosphatase mutant displayed a modest decrease in the growth rate, Δ*ushA*Δ*cpdB* reached the same final optical density as the wild type control after approximately 70 hours of growth (Figure 7). Therefore, we concluded that one (or more) of the remaining three putative nucleotidases (*phoX*, VCA0545, or VCA0608) was responsible for the ability the bacterium to grow in the absence of *ushA* and *cpdB*. To address this hypothesis, we tested the triple mutants (Δ*ushA*Δ*cpdB* combined with an additional phosphatase mutant) for growth on eDNA as the sole source of phosphate. Indeed, we found that the additional deletion of *phoX*, but not VCA0545 or VCA0608, resulted in a growth phenotype nearly identical to the Δ5 phosphatase mutant strain (Figure 8). By day six, the Δ*ushA*Δ*cpdB*Δ*phoX* mutant exhibited slight growth that was not apparent in the Δ5 phosphatase mutant. The amount of growth varied between replicates and experiments, and is not immediately obvious in the curve presented in Figure 8. We did not determine if the triple mutant could continue to grow and

reach wild type turbidity. We hypothesized that this slight growth is due to activity of either VCA0545 and/or VCA0608. These data support that UshA, CpdB, and PhoX are the major phosphatases that allow *V. cholerae* to utilize eDNA as a source of phosphate.

CpdB, but not UshA, is activated by phosphate limitation

We predicted that the nucleotide phosphatases would be induced under phosphate limitation, when they would be required for phosphate scavenging by *V. cholerae*. Indeed, *phoX* is a low phosphate induced gene (von Kruger *et al.*, 2006). We performed qRT-PCR experiments to determine if *ushA* and *cpdB* are also expressed upon transition to phosphate limiting conditions. Although we were able to induce expression of *phoX* after incubation of bacteria in a no phosphate medium, we did not detect increased transcription of *ushA* or *cpdB* when compared with the high phosphate condition (data not shown and Supplementary experimental procedures).

To determine if UshA or CpdB nucleotidase activity is regulated by phosphate conditions, we performed nucleotidase assays using bacteria that were incubated in either high phosphate or no phosphate MOPS-glucose media. The wild type rate of phosphate removal from 5’dAMP and 5’dGMP was very similar under high phosphate and no phosphate conditions (Figure 9a–b). Conversely, 3’nucleotidase activity was induced in the wild type by phosphate limitation (Figure 9c–d). Incubation of the bacteria in high phosphate rendered 3’nucleotidase activity against 3’dGMP undetectable, but slight activity still remained against 3’AMP. CpdB accounts for all 3'nucleotidase activity under both high phosphate and no phosphate environments, as the deletion strain did not exhibited any detectable phosphate release under either condition. Since we did not test activity against 3'dAMP or 3'GMP, we cannot say whether CpdB 3'nucleotidase activity under high phosphate is specific to the adenosine nucleotide or ribo-nucleotides in general. These results explain why the 5’dGMP nucleotidase activity presented in Figure 6 is unexpectedly low based on the growth assays in Figure 5; the medium used in that experiment, LB, is a high phosphate environment. From these experiments we conclude that CpdB activity is phosphate regulated due to some post-transcriptional process.

Discussion

Being comprised of phosphate, sugars, and nucleic acids, DNA is a rich source of phosphate, carbon, and nitrogen. Several bacterial species, including *V. cholerae*, are able to utilize eDNA as a source of nutrients (Mulcahy *et al.*, 2010; Pinchuk *et al.*, 2008; Rittmann *et al.*, 2005; Sebastian and Ammerman 2011; Seper *et al.*, 2011; Trulzsch *et al.*, 2001). The utilization of eDNA as a source of phosphate requires break down of the DNA strands into nucleotides, removal of the phosphate from the nucleoside, and uptake of the phosphate into the cell via dedicated transporters (*e.g.* Pit and Pst/PhoU). Under phosphate limiting conditions, *V. cholerae* expresses and secretes an exo- and endo-nuclease, Xds and Dns, respectively (McDonough *et al.*, 2014; Seper *et al.*, 2011). A mutant deleted for both of these nuclease genes exhibits a severe growth defect when eDNA is supplied as the sole source of phosphate (Seper *et al.*, 2011). This suggests that if there is an additional secreted nuclease produced by *V. cholerae*, it does not significantly contribute to the acquisition of

phosphate from eDNA under *in vitro* conditions. While the cytoplasmic-uptake machinery for phosphate is well characterized in *V. cholerae*, no phosphatases involved in utilization of DNA/nucleotides as sources of phosphate had been described in *V. cholerae*. Therefore, the goal of this work was to identify the phosphatases involved in this phenotype. A model of our findings is presented in Figure 10. In summary, we identify UshA, CpdB, and PhoX as the major phosphatases that allow *V. cholerae* to utilize eDNA as a source of phosphate.

Although we have focused on describing *V. cholerae* UshA and CpdB as 5’ and 3’nucleotidases, respectively, we have not determined if these proteins carry the bifunctional activities (UDP-sugar hydrolase and 2’3’phosphodiesterase, respectively) as described in other organisms. Our bioinformatic searches for phosphatases identified two additional genes: VCA0545 and VCA0608. VCA0545 is homologous to UshA in *V. cholerae* and in other species. VCA0608 is annotated as a dUMP phosphatase, and thus may be specific for this ribo-nucleotide intermediate. We were unable to verify the phosphatase activities of these two proteins, however, as described below, our phenotypic data implicate one or both of these proteins in residual phosphatase activity.

A quintuple mutant (all 5 putative phosphatases deleted) demonstrated an inability to use eDNA as a phosphate source after seven days of incubation in the growth medium. Similarly, a triple mutant, Δ*ushA*Δ*cpdB*Δ*phoX*, was a near phenocopy of the Δ5 phosphatase mutant. However, slight growth of the triple mutant was observed by day six, suggesting that another phosphatase, likely VCA0545 or VCA0608, provides minimal phosphatase activity against eDNA or nucleotides. While we can easily attribute the role of UshA and CpdB in eDNA growth to their nucleotidase activities, the role of PhoX is less clear. *V. cholerae* PhoX was previously demonstrated to be inactive against 5’AMP or 5’ATP, and thus presumed inactive against all nucleotides (Roy *et al.*, 1982). Although these authors did not test PhoX for phosphatase activity against other nucleotides, it seems unlikely that the generally non-specific phosphatase would act on some nucleotides but not others. However, our data clearly suggest that PhoX must be active as a phosphatase against either a subset of nucleotides that we were unable to test (3’dAMP or 3’dCMP) or DNA. Further biochemical analysis of PhoX could elucidate the precise role that the protein plays in eDNA growth.

While expression of *phoX* is induced by phosphate limitation (von Kruger *et al.*, 2006), we did not detect increased transcription of *ushA* or *cpdB*. However, we did observe an increase in CpdB nucleotidase activity after incubation of the bacteria in media lacking phosphate. Thus, we conclude that CpdB is likely post-transcriptionally regulated by phosphate. For example, either the protein level or activity of the enzyme is induced by phosphate limitation.

Despite its universal role in phosphate assimilation, expression of *ushA* in relation to phosphate concentrations is not consistent between other bacterial species. For example, *ushA* is expressed under low phosphate conditions in *C. glutamicum* and this expression is regulated through the phosphate starvation regulator, PhoS (Kocan *et al.*, 2006; Rittmann *et al.*, 2005). Alternatively, *E. coli ushA* is not regulated by phosphate conditions or PhoB, a homolog of PhoS (Burns and Beacham 1986). The *V. cholerae ushA*, which encodes a protein more similar to *E. coli* than to *C. glutamicum*, is likely not regulated by phosphate.

However, it is important to note that there may be small changes in transcription of *ushA* that we are unable to detect with the qRT-PCR assay.

Regulation of *cpdB* in other organisms has not been thoroughly studied; however the gene is not induced by phosphate limitation in *Salmonella* Typhimurium (Kier *et al.*, 1977). Alternatively, *cpdB* of both *Salmonella* Typhimurium and *Yersinia* spp. are under control of carbon catabolite repression (Kier *et al.*, 1977; Trulzsch *et al.*, 2001). A cAMP-CRP binding site has been identified in the *cpdB* promoter of *Y. entericolitica*, and CpdB allows growth of this species on cAMP as the sole source of carbon (Trulzsch *et al.*, 2001). Using the online promoter prediction tool, Softberry BRPOM (Solovyev and Salamov 2011), we identified two putative CRP binding sites in the *V. cholerae cpdB* promoter. Thus, it will be interesting to see if *cpdB* from this organism is carbon catabolite repressed, while phosphate levels control the enzymatic activity.

The physiological relevance of phosphate acquisition from eDNA by *V. cholerae* is unclear. Within a human host, *V. cholerae* may encounter eDNA in the form of NETs secreted by attacking neutrophils. Indeed, the secreted nucleases, Xds and Dns, were shown to be important in defending *V. cholerae* from NET attack by breaking down the structural DNA of the NET (Seper *et al.*, 2013). Since we have shown that the small intestine of an infant mouse is a phosphate limiting environment, we hypothesize that DNA from NETs can also serve as a source of phosphate for the pathogen (McDonough *et al.*, 2014).

DNA is a known structural component of *V. cholerae* biofilms, which form in the aquatic environment where nutrients are often limiting. In the context of a biofilm, Xds and Dns were demonstrated as important for allowing individual bacteria to degrade the DNA structure and escape (Seper *et al.*, 2011). Since DNA is a rich source of phosphate, it seems likely that the organism would take advantage of the released nucleotides and consume them as nutrients. Therefore, we hypothesize that the genes involved in acquiring nutrients from eDNA (*e.g. ushA*, *cpdB*, and *phoX*) would be important for survival of *V. cholerae* in a biofilm.

V. cholerae biofilms in the aquatic environment may develop on chitinous surfaces, *eg.* exoskeletons of copepods (Meibom *et al*., 2005). Under these conditions, *V. cholerae* is naturally transformable; chitin induces expression of the DNA uptake machinery, such as the competence pilus (Meibom *et al*., 2004; Meibom *et al.*, 2005). The process of DNA uptake via natural transformation has been suggested as a mechanism of nutrient acquisition in other organisms (Sinha *et al.*, 2013), although this hypothesis is highly debated (Johnston *et al.*, 2014). Considering that upon transport into the cytoplasm by competence machinery, single stranded DNA is very quickly coated with single-stranded binding protein (SSB), it seems unlikely that this DNA could be accessed by degradation enzymes that would allow its use as a source of nutrients (Dubnau 1999). The strand of DNA not taken up by the cell is degraded into nucleotides, providing a source of energy for the transport of the intact strand across the inner membrane. Thus it is possible that this broken down strand is further degraded by UshA, CpdB, and/or PhoX into nucleoside and phosphate and used as nutrients. Until further work has been complete it will be unclear if *V. cholerae* natural competence is tied with nutrients acquisition.

The source of eDNA within the *V. cholerae* biofilm is unknown. *Neisseria* spp. are known to secrete chromosomal DNA, a phenotype that has been hypothesized as important in both natural competence as well as biofilm formation (Hamilton *et al.*, 2005; Lappann *et al.*, 2010). Like *Neisseria* spp., *V. cholerae* might actively secrete DNA for use in the biofilm matrix. Alternatively, the eDNA isolated from the biofilm matrix of *V. cholerae* may be released upon cell lysis. Aside from the eDNA present in *V. cholerae* biofilms, the aquatic environment contains a great deal of eDNA. Reported concentrations of eDNA in the aquatic environment range from picomolar to micromolar amounts (Bjorkman and Karl 2005; Lorenz and Wackernagel 1994). Although we have used a concentration of eDNA approximately 10-fold less than Seper and colleagues (2011), we did not attempt to titrate the concentration of eDNA to determine whether picomolar or micromolar amounts of eDNA are enough to support survival of *V. cholerae*. However, the DNA found in biofilms or in NETS is likely more concentrated, and may be similar to the concentration of DNA that we have used.

In summary, we have described results that demonstrate the requirement of a 5’ and 3’nucleotidase (UshA and CpdB, respectively), for the ability of *V. cholerae* to grow using nucleotides as a phosphate source. To our knowledge, we are the first to show that *V. cholerae* can utilize both 5’ and 3’nucleotides as sources of phosphate, as well as being the first to describe the ability of any bacterium to survive using 3’nucleotides as a source of phosphate. Interestingly, we have also shown that the organism is unable to use 3’CMP. We demonstrated that UshA and CpdB, work together with alkaline phosphatase, PhoX, to release phosphate from eDNA. While *phoX* is induced by phosphate limitation, *ushA* and *cpdB* do not appear to be. However, CpdB enzymatic activity is increased in phosphate depleted medium. Prior to our work, UshA and CpdB had not been characterized in *V. cholerae* or any of the *Vibrionaceae*, and PhoX was considered to be inactive against all nucleotides (Roy *et al.*, 1982) – a result that this work throws into question. Additionally, this is the first demonstration of a 2’3’cyclic phosphodiesterase/3’nucleotidase, CpdB, being involved in phosphate acquisition from DNA.

Experimental Procedures

Media and bacterial strains

Bacterial strains were propagated in LB broth with aeration or on LB agar at 37°C, unless otherwise noted. When indicated bacteria were grown in MOPS-glucose minimal media [1× MOPS salts (40 mM MOPS pH 7.4 (3 –(N-morpholino)propanesulfonic acid) (Sigma Aldrich), 4 mM tricine, 0.1 mM $FeSO_4$•$7H_2O$, 9.5 mM NH_4Cl, 0.28 mM KCl, 0.53 mM $MgCl_2$•$6H_2O$, and 50 mM NaCl); 1× NRES (25 mM of each of the amino acids N, R, E, and S); 1× trace metals (0.005% $MgSO_4$, 0.0005% $MnCl_2$•$4H_2O$, 0.0005% $FeCl_3$, and 0.0004% nitrilotriacetic acid); and 0.5% glucose], supplemented with various sources of phosphate. Unless otherwise noted, antibiotics were used at the following concentrations: 100 μg ml^{-1} streptomycin (Sm), 50 μg ml^{-1} ampicillin (Ap), 2 μg ml^{-1} chloramphenicol (Cm), 50 μg ml^{-1} spectinomycin (Sp), and 50 μg ml^{-1} kanamycin (Kn). Addition of 0.5 mM IPTG to broth was used to induce transcription from the P_{tac} promoter.

Strain construction

Bacterial strains and plasmids used are listed in Table 1. PCR primers used are listed in Table 2. All *V. cholerae* strains were constructed using standard molecular techniques in an Sm resistant derivative of the clinical O1 El Tor isolate E7946 (Mekalanos 1983). Unless stated otherwise, all mutations generated in this study were confirmed by Sanger sequencing by the Tufts University Core Facility or by Eton Bioscience (Charlestown, MA). Plasmids were maintained in *E. coli* DH5αλ*pir*. The donor strain, *E. coli* MFD*pir*, was used for conjugative transfer of plasmids (Ferrieres *et al.*, 2010).

The single mutants – Δ*ushA* and Δ*nupC*– were constructed using the Trans-FLP method, which utilizes the natural transformability of chitin-grown *V. cholerae* and Flp-recombination, as described previously. Briefly, a gene of interest is replaced with a selectable FRT cassette, which is flipped out of the genome by the Flp recombinase carried on pBR-flp (Blokesch 2012; De Souza Silva and Blokesch 2010; McDonough *et al.*, 2014).

Natural co-transformation as previously described was used to make the single mutants: Δ*cpdB*, ΔVCA0545, VCA0608 (Dalia *et al.*, 2014). The selectable marker used in this construction was pBAD33kan isolated from *E. coli* TG1 cells. The PCR constructs used for transformation resulted in the exchange of the desired open reading frame for a FRT scar (GAAGCAGCTCCAGCCTACA), leaving only the start and stop codon of the deleted gene. Tranformants were selected on LB plates with 75 μg ml^{-1} Kn and subsequently screen by MASC PCR in order to confirm the genotype of each transformant at all loci of interest (Wang and Church 2011a). The Δ*ushA* Δ*cpdB* mutant was constructed using natural co-transformation and Δ*ushA* as the parental strain. A second round of natural co-transformation on this strain was used to make the Δ*ushA* Δ*cpdB* ΔVCA0545 and Δ*ushA* Δ*cpdB* ΔVCA0608 mutants. The triple mutant Δ*ushA* Δ*cpdB* Δ*phoX* was constructed by first using Trans-Flp to delete *ushA* in the *phoX* deletion background. We were unable to obtain a mutant with the FRT cassette flipped out, so *ushA* is replaced by the FRT-Spec-FRT cassette in this strain. Secondly, natural co-transformation was used to replace *cpdB* with a FRT scar. The Δ5 phosphatase mutant was constructed by using the Δ*ushA* (FRT-spec-FRT) Δ*phoX* parental strain and two rounds of natural co-transformation to additionally delete *cpdB*, VCA0545, and VCA0608.

The complementation plasmids pMMB67EH-*ushA* and pMMB67EH-*cpdB* were constructed in the pMMB67EH vector. *V. cholerae ushA* was amplified from E7946 genomic DNA using the primers pMMB-ushA F and pMMB ushA R. Likewise, *cpdB* was amplified from E7946 genomic DNA using the primers pMMB-cpdB F and pMMB-cpdB R.The PCR fragments were digested with SacI and PstI restriction enzymes and then ligated into pMMB67EH that had been similarly digested. pMMB67EH-*ushA*, pMMB67EH-*cpdB*, and pMMB67EH were cloned in *E. coli* DH5λ*pir*, and transferred into the conjugation donor strain *E. coli* MFD*pir* (Ferrieres *et al.*, 2010). The plasmids were moved into *V. cholerae* using filter mating and the exconjugates were selected by plating on Sm and Ap. The primers pMMB-F and pMMB-R were used to screen for isolates carrying the expression vector and insert.

Phosphatase screen

The mTn*10* library was constructed in Δ*phoX* using pDL1098 as described previously (McDonough *et al.*, 2014). Aliquots of the library were thawed, diluted to 10^{-5} in LB, and 225 μl were plated on 150 mm LB plates supplemented with 100 μg ml^{-1} Sp and 40 μg ml^{-1} XP. Plates were incubated overnight at 37°C and white colonies were identified (7/~40,000) and colony purified on the same medium. Genomic DNA was prepped from each white colony and arbitrary primed PCR followed by sequencing of the PCR product was used to determine the location of each mTn*10* insertion (Hava and Camilli 2002). Briefly, two rounds of PCR were performed using the primers 1) Arb1/olj363 and 2) Arb2/olj386. For PCR 1 the following program was used: 95°C for 5 min, followed by 6 rounds of 95°C for 30 sec, 30°C for 30 sec, and 72°C for 1 min; followed by 30 rounds of 95°C for 30 sec, 45°C for 30 sec, and 72°C for 1 min. For PCR 2 the following program was used: 95°C for 5 min, followed by 35 rounds of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. PCR products were cleaned and sent for sequencing using the primer olj386.

Phosphate growth curves

Strains were struck on LB Sm plates and grown overnight at 37°C. Three single colonies per culture were used to inoculate 2 ml cultures of LB, and grown for 4 hours at 37°C with aeration. Next, strains were back-diluted to an OD_{600} ~0.05 and grown to mid-exponential phase (OD_{600} ~0.5) in 3 ml MOPS-glucose medium supplemented with 10 mM KH_2PO_4. Bacteria were washed twice in MOPS-glucose medium (no phosphate) and inoculated into the growth curve test cultures.

For testing growth in minimal medium plus eDNA, strains were inoculated into 2 ml cultures of MOPS-glucose medium supplemented with the desired source of phosphate (either KH_2PO_4, DNA, or no phosphate). Culture tubes were grown at 37°C with aeration and OD_{600} readings were taken through the glass tube after blanking the spectrophotometer with the appropriate medium. New glass tubes were always used to avoid misreading of the optical density due to scratches in the glass, as well as to avoid phosphate contamination from the phosphoric acid used to wash the dishes.

The source of eDNA used in these growth experiments was sheared salmon sperm DNA (Life Technologies). According to agarose gel electrophoresis analysis, the DNA strands range from ~50 basepairs to 500 basepairs in length. The concentration of 0.5 mM used in these experiments refers to the total amount of phosphate molecules in the DNA, and not the molarity of the DNA. The stock of 10 mg ml^{-1} sheared salmon sperm DNA was calculated to be 25.5 mM phosphate.

For growth in minimal medium plus nucleotides, strains were pre-grown and washed as described for eDNA growth curves. Following this, strains were inoculated into 200 μl MOPS-glucose medium supplemented with the desired source of phosphate (either KH_2PO_4, nucleotides, or nothing). Nucleotides were added at a concentration of 0.1 mM total phosphate. The cultures were grown in 96-well plates at 37°C with aeration using the BioTek Synergy Plate Reader. Optical density readings were measured and recorded every 15 min using the Gen5 Data Analysis software. All nucleotides were obtained from Santa

Cruz Biotechnology, except 5'dAMP, 5'dCMP, and 5'dGMP which were obtained from Sigma-Aldrich. The nucleotides were obtained in powder form and resuspended in pure H_2O, except 5'dAMP, which was resuspended in 200 mM NaOH.

Nucleotidase assays

Single colonies were used to inoculate 10 ml LB cultures, which were grown at 37°C with aeration to an optical density of ~0.5. For each replicate, the equivalent of 10 ml of OD_{600} =0.5 culture was centrifuged in a 15 ml conical tube at 4,500 × *g* for 20 min at room temperature. The supernatants were removed and the cultures were resuspended in 1 ml 10 mM Tris HCl pH 7.5. The cultures were spun again and the pellets were resuspended in 0.5 ml 10 mM Tris HCl pH 7.5. At this point cells were lysed by transferring the cells to 2 ml eppendorf tubes and sonicating for 1 min with 50% amplitude, ½ sec on and ½ sec off using a high-intensity cuphorn sonifier (Branson). Cell lysates were clarified by spinning the tubes at 8,000 × *g* for 10 min at 4°C.

For nucleotidase assays performed under defined phosphate conditions, bacteria were pre-grown in LB and back diluted to OD_{600}=0.05 in 10 mM phosphate MOPS-glucose medium. After reaching OD_{600}=0.4–0.6, the bacteria were washed twice in MOPS-glucose medium lacking phosphate, and resuspended in either no phosphate or 10 mM phosphate MOPS-glucose medium. Bacteria were incubated for 2 hours at 37°C. The equivalent of 1 ml at OD_{600}=0.6 bacteria were washed once in 10 mM Tris pH 7.5, resuspended in 100 µl of the same buffer and used directly in the nucleotidase assay.

Nucleotidase assays were performed as described previously with a few modifications (Edwards *et al.*, 1993). 100 µl of the cells lysates were mixed with 890 µl of assay buffer (150 µl 0.5 M Sodium Acetate pH 6.0, 30 µl 150 mM $CoCl_2$, 30 µl 480 mM $CaCl_2$, and water to a final volume of 890 µl) and equilibrated to 37°C for 5 min. The nucleotidase reaction was started by the addition of 10 µl 100 mM nucleotide substrate and samples were immediately mixed by vortexing and placed at 37°C. At times 0, 5, 10, and 15 min after addition of the substrate, 150 µl samples of each reaction were removed and transferred to eppendorf tubes with 100 µl 0.1 N HCl and placed on ice to stop the reaction. Once all samples were acquired, the tubes were centrifuged at 16,000 × *g* for 5 min at 4°C to pellet cell debris. Subsequently, 60 µl of the supernatant was mixed with 140 µl of the development reagent (1 part 10% ascorbic acid, 6 parts 0.42% Ammonium molybdate in 1 N H_2SO_4). Samples were incubated at 45°C for 20 min, after which 150 µl of each sample was transferred to a 96-well plate and the absorbance at 820 nm was measured using a BioTek Synergy Plate Reader.

To convert the absorbance readings to nmoles of phosphate released, a standard curve was performed. Five-fold serial dilutions of KH_2PO_4 corresponding to 1000, 200, 40, 8, 1.6, or 0 pmoles of phosphate were mixed with the assay buffer and then mixed with the development reagent and incubated as described above. After the absorbance was measured, the readings were plotted against starting concentration and the slope corresponded to the conversion factor (*i.e.* absorbance readings in subsequent assays were converted to pmoles released by the slope). The standard curve was performed twice, in duplicate each time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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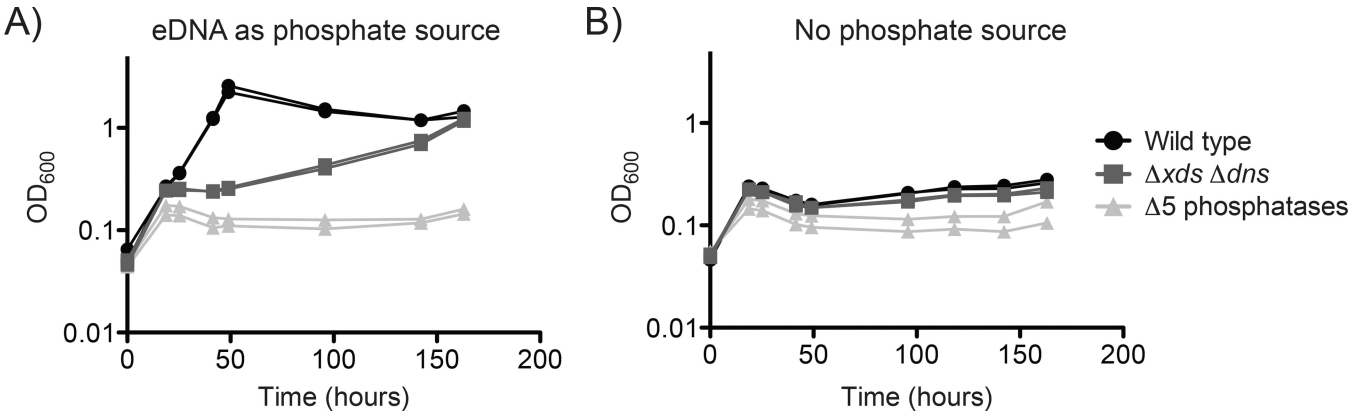

Figure 1. Deletion of 5 putative phosphatases hinders growth on eDNA as a source of phosphate

Growth on eDNA was assessed using mid-exponential phase bacteria, which were washed twice before putting in to the test conditions. The growth medium used was MOPS-glucose supplemented with either A) sheared salmon sperm DNA consisting of 0.5 mM phosphate or B) no phosphate. The Δ5 phosphatases mutant is: Δ*phoX* Δ*ushA* Δ*cpdB* Δ*yjjG* ΔVCA0545. Shown is a representative graph of the mean of two biological replicates assayed on the same day. The growth assay was performed two times, with a total of four biological replicates; each experiment exhibited the same results.

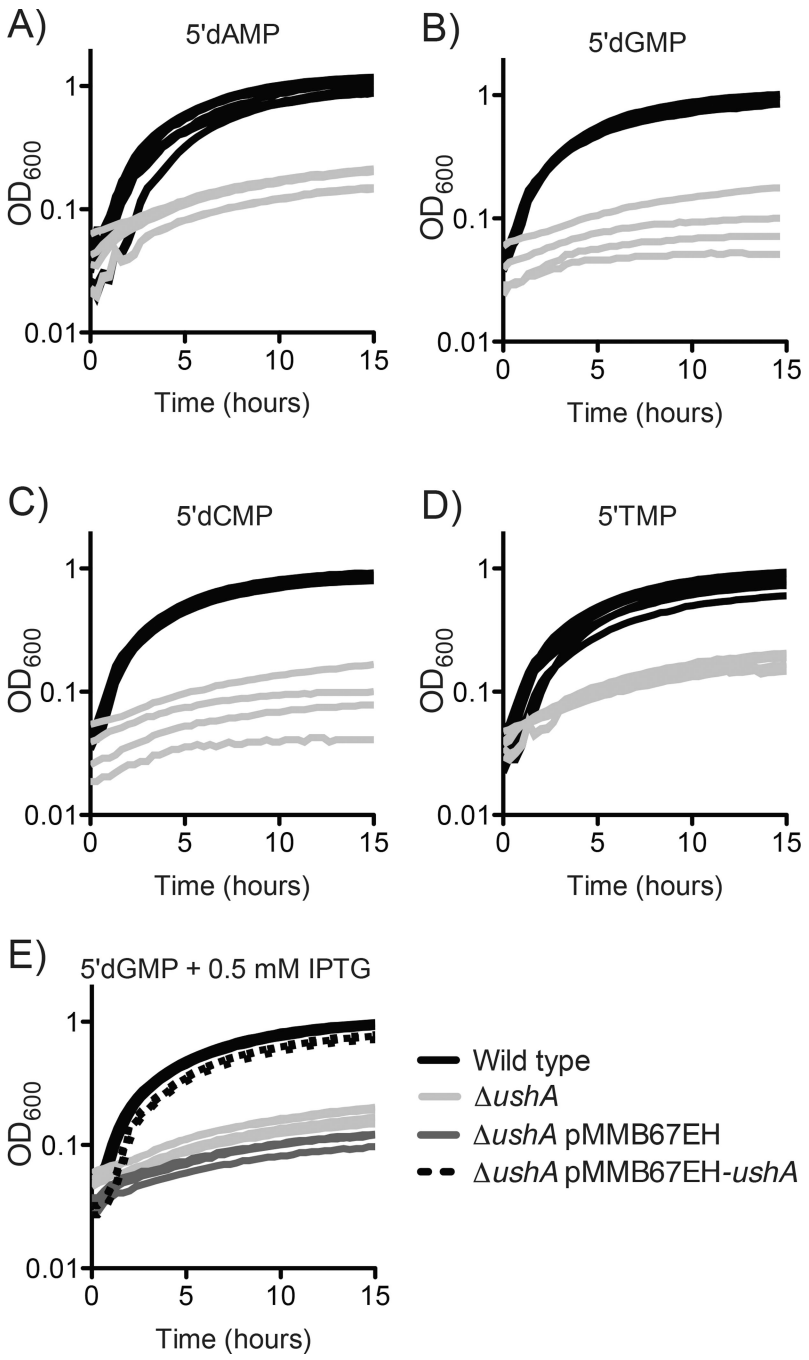

Figure 2. *ushA* is required for growth on all 5' nucleotides when supplied as sources of phosphate

A) – D) Bacteria were pre-grown to mid-exponential phase in MOPS-glucose minimal medium, supplemented with 10 mM KH_2PO_4. Cultures were washed two times in MOPS medium containing no phosphate and inoculated into 200 μl MOPS-glucose medium with either A) 0.1 mM 5'dAMP, B) 0.1 mM 5'dGMP, C) 0.1 mM 5'dCMP, or D) 0.1 mM 5'TMP. Strains were grown at 37°C with aeration in a 96-well plate. The mean of at least four biological replicates, assayed on at least two different days, is shown for all growth assays. Double times are reported in Table 1.

E) For complementation, *ushA* was expressed from the IPTG-inducible P_{tac} promoter carried on the pMMB67EH plasmid. After pre-growth and washing as described above, strains were inoculated into 200 μl MOPS-glucose medium with 0.1 mM 5'dGMP + 0.5 mM IPTG. The strains harboring the expression vector were grown in the presence of Ap. Strains were grown at 37°C with aeration in a 96-well plate. The mean of at least four biological replicates, assayed on at least two different days, is shown.

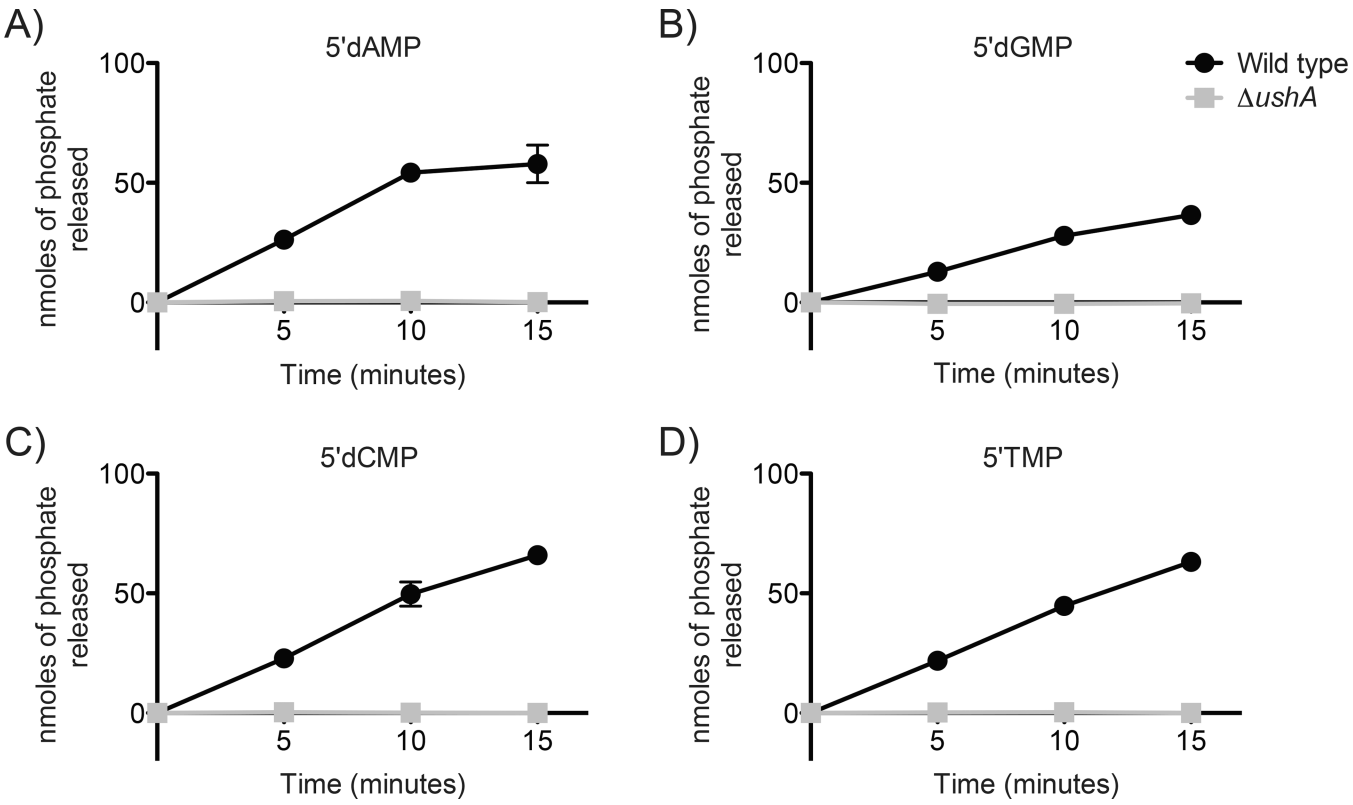

Figure 3. UshA is required for 5' nucleotidase activity

Wild type and Δ*ushA* strains were grown to an OD_{600} of ~0.5 in 10 ml LB cultures. Cultures were washed once in 10 mM Tris pH 7.5 and lysed by sonication. Lysates were mixed with a final concentration of 1 mM A) 5'dAMP, B) 5'dGMP, C) 5'dCMP, or D) 5' TMP. At 0, 5, 10, and 15 min after addition of the substrate, aliquots of the reaction were removed and mixed with 0.1 N HCl to prevent further enzymatic activity. After all samples were collected, cellular debris was removed by centrifugation and the supernatants were incubated with the ammonium molybdate solution (1% ascorbic acid and 1 N H_2SO_4) at 45°C for 20 min. Nanomoles of phosphate released by enzymatic activity was determined by measuring the OD at 820nm and converting to nmole through use of a standard curve. The mean and standard error of at least three replicates, assayed on at least two different days, are shown for each assay.

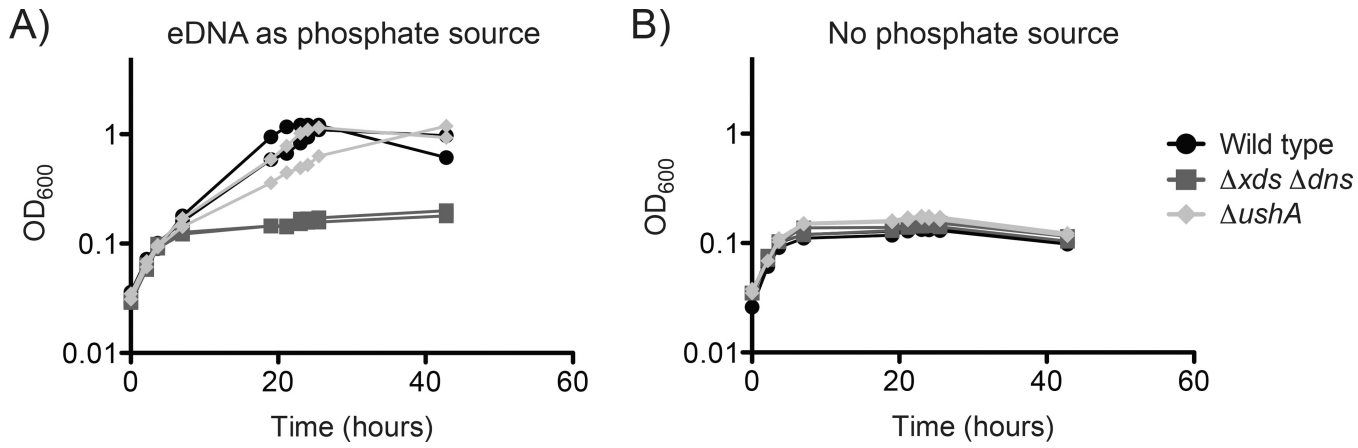

Figure 4. *ushA* is not required for growth on eDNA as a source of phosphate

Growth on eDNA was assessed using mid-exponential phase bacteria, which were washed twice before putting in to the test conditions. The growth medium used was MOPS-glucose supplemented with either A) 0.5 sheared salmon sperm DNA consisting of 0.5 mM phosphate or B) no phosphate. Shown is a representative graph of the mean of two biological replicates assayed on the same day. The growth assay was performed four times, with a total of eight biological replicates; each experiment exhibited the same results.

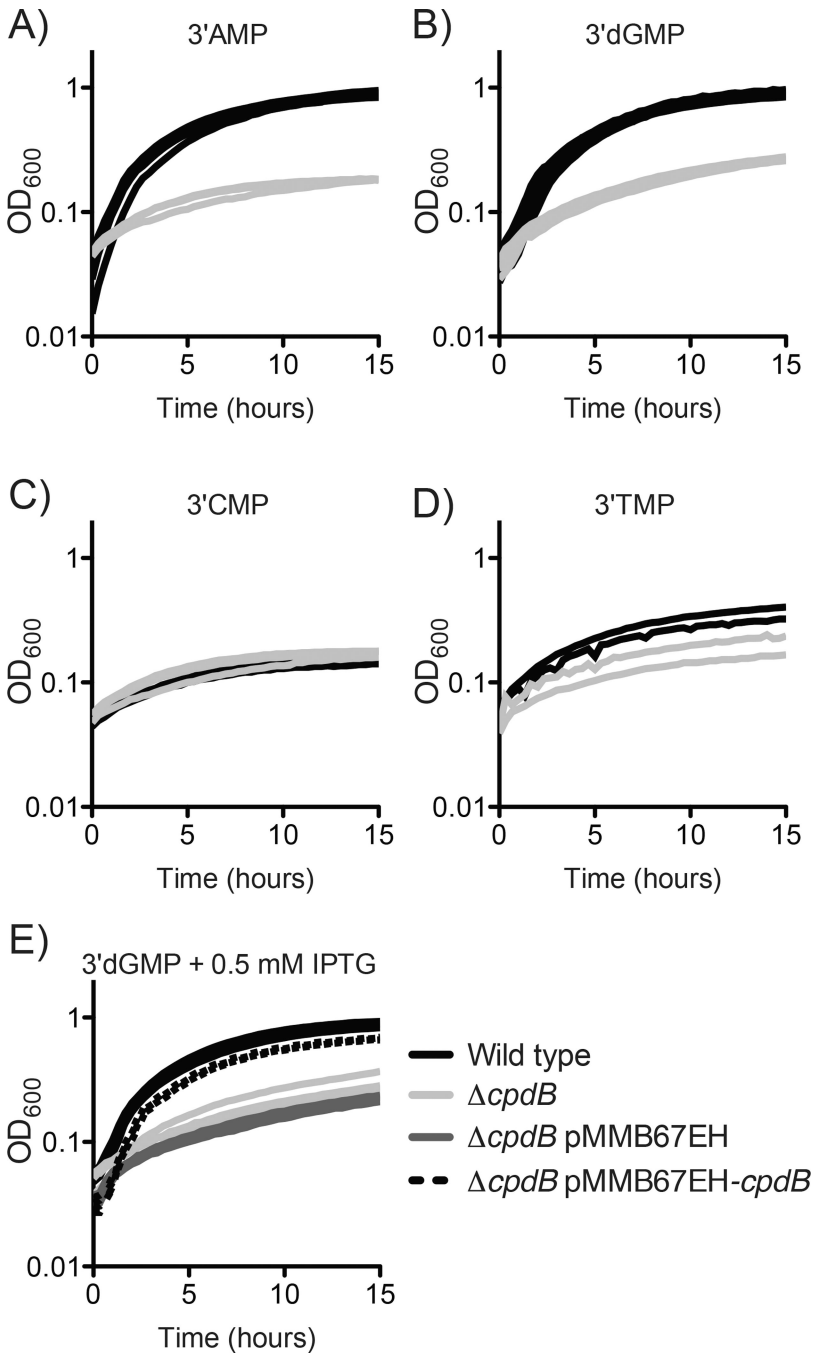

Figure 5. *cpdB* is required for growth on 3'AMP and 3'dGMP when supplied as sources of phosphate

A) – D) Bacteria were pre-grown to mid-exponential phase in MOPS-glucose minimal medium, supplemented with 10 mM KH_2PO_4. Cultures were washed twice in MOPS medium containing no phosphate and inoculated into 200 µl MOPS-glucose medium with either A) 0.1 mM 3'AMP, B) 0.1 mM 3'dGMP, C) 0.1 mM 3'CMP, or D) 0.1 mM 3'TMP. Strains were grown at 37°C with aeration in a 96-well plate. Each line represents the mean of at least two biological replicates. Double times are reported in Table 1.

E) For complementation, *cpdB* was expressed from the IPTG-inducible P_{tac} promoter carried on the pMMB67EH plasmid. After pre-growth and washing as described above, strains were inoculated into 200 μl MOPS-glucose medium with 0.1 mM 3'dGMP + 0.5 mM IPTG. The strains harboring the expression vector were grown in the presence of Ap. Strains were grown at 37°C with aeration in a 96-well plate. The mean of at least four biological replicates, assayed on at least two different days, is shown.

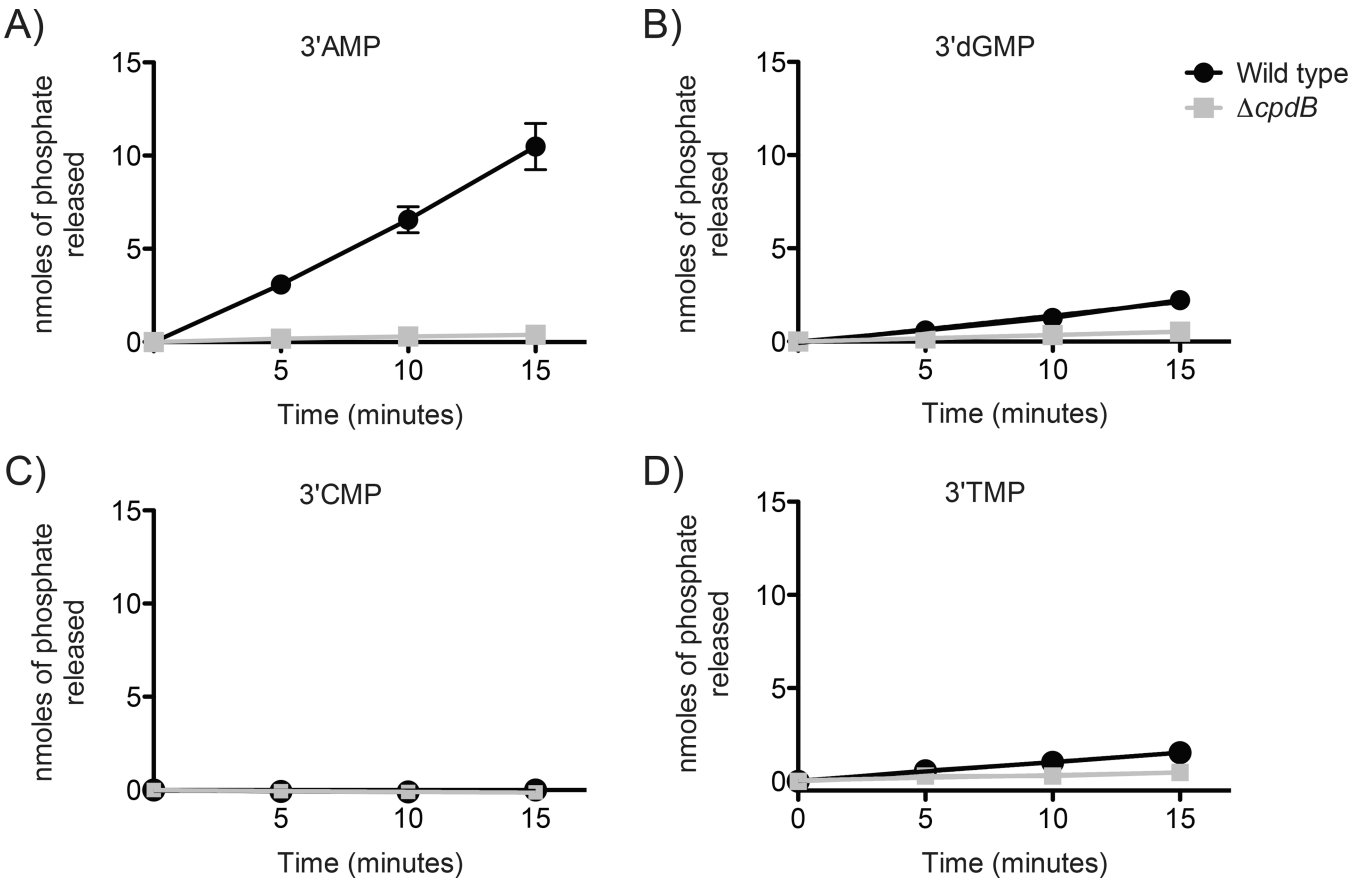

Figure 6. CpdB is required for 3' nucleotidase activity on certain nucleotides

Wild type and Δ*cpdB* strains were grown to an OD_{600} of ~0.5 in 10 ml LB cultures. Cultures were washed once in 10 mM Tris pH 7.5 and lysed by sonication. Lysates were mixed with a final concentration of 1 mM **A)** 3'AMP, **B)** 3'dGMP, **C)** 3'CMP, or **D)** 3' TMP. At 0, 5, 10, and 15 min after addition of the substrate, aliquots of the reaction were removed and mixed with 0.1 N HCl to prevent further enzymatic activity. After all samples were collected, cellular debris was removed by centrifugation and the supernatants were incubated with the ammonium molybdate solution (1% ascorbic acid and 1 N H_2SO_4) at 45°C for 20 min. Nanomoles of phosphate released by enzymatic activity was determined by measuring the OD at 820nm and converting to nmole through use of a standard curve. The mean and standard error of at least two replicates are shown for each assay.

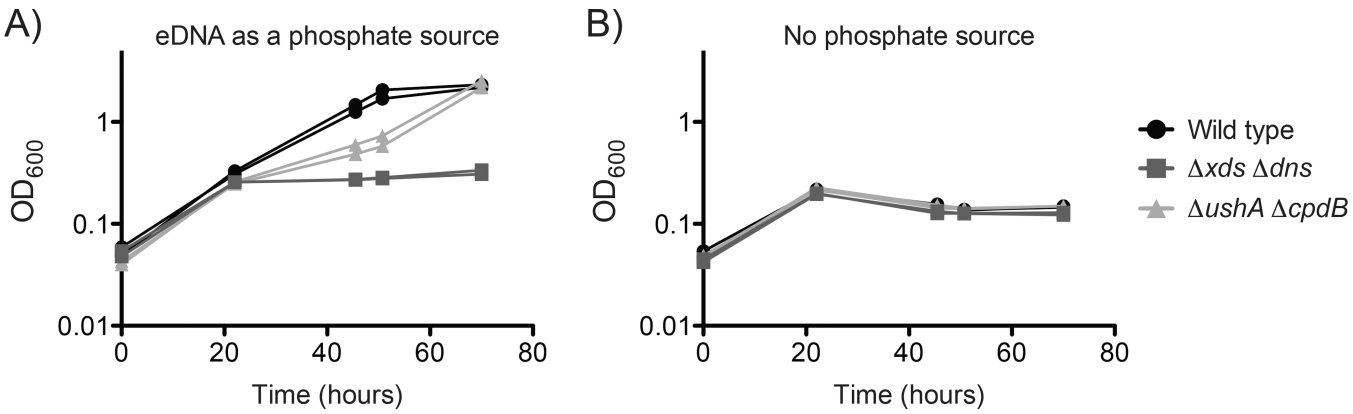

Figure 7. Deletion of *cpdB*, together with *ushA*, does not abolish growth on eDNA as a sole source of phosphate

This growth assay was performed using mid-exponential phase bacteria, which were washed twice before putting into the test conditions. The growth medium used was MOPS-glucose supplemented with either A) sheared salmon sperm DNA consisting of 0.5 mM phosphate or B) no phosphate source. The experiment was performed three times with a total of 5 biological replicates. The mean of two biological replicates assayed in the same experiment is shown.

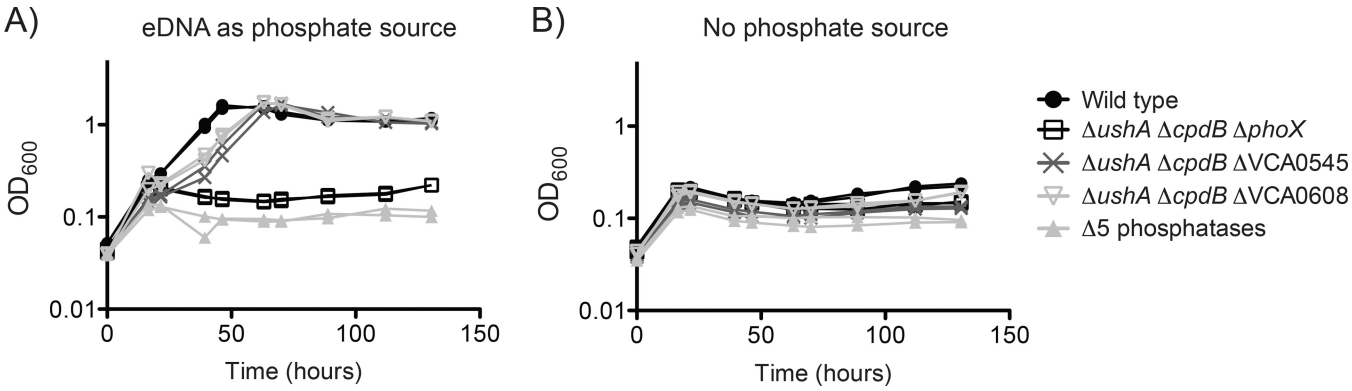

Figure 8. Deletion of *ushA*, *cpdB*, and *phoX* mimics the delta 5 phosphatase mutant

This growth assay was performed using mid-exponential phase bacteria, which were washed twice before putting into the test conditions. The growth medium used was MOPS-glucose supplemented with either A) sheared salmon sperm DNA consisting of 0.5 mM phosphate or B) no phosphate source. The experiment was performed twice with two biological replicates each time. The mean of two replicates from the same experiment is shown.

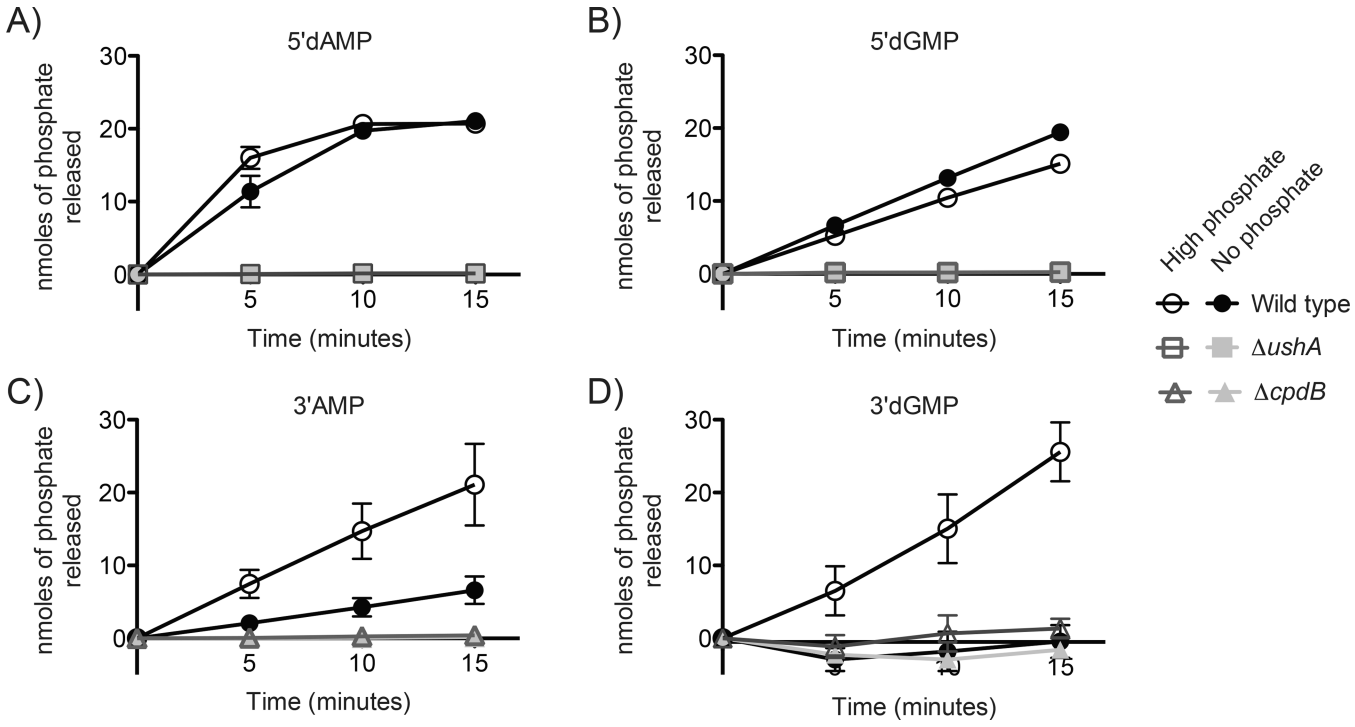

Figure 9. 3'nucleotidase activity is induced by phosphate limitation

Bacterial cultures were grown to mid-exponential phase in MOPS-glucose medium supplemented with 10 mM KH_2PO_4, washed twice in no phosphate MOPS-glucose, and resuspended into 2 test conditions: MOPS-glucose medium supplemented with 10 mM KH_2PO_4 or no phosphate. After 2 hours of incubation at 37°C in the test conditions, the bacteria were washed once in 10 mM Tris pH 7.5, and resuspended in 100 µl of the same buffer. The cells were mixed with assay buffer and a final concentration of 1 mM **A)** 5'dAMP, **B)** 5'dGMP, **C)** 3'AMP, or **D)** 3' dGMP. At 0, 5, 10, and 15 min after addition of the substrate, aliquots of the reaction were removed and mixed with 0.1 N HCl to prevent further enzymatic activity. After all samples were collected, cellular debris was removed by centrifugation and the supernatants were incubated with the ammonium molybdate solution (1% ascorbic acid and 1 N H_2SO_4) at 45°C for 20 min. Nanomoles of phosphate released by enzymatic activity was determined by measuring the OD at 820nm and converting to nmole through use of a standard curve. The mean and standard error of four replicates (5'dGMP and 3'AMP) or two replicates (5'dAMP and 3'dGMP) are shown for each assay.

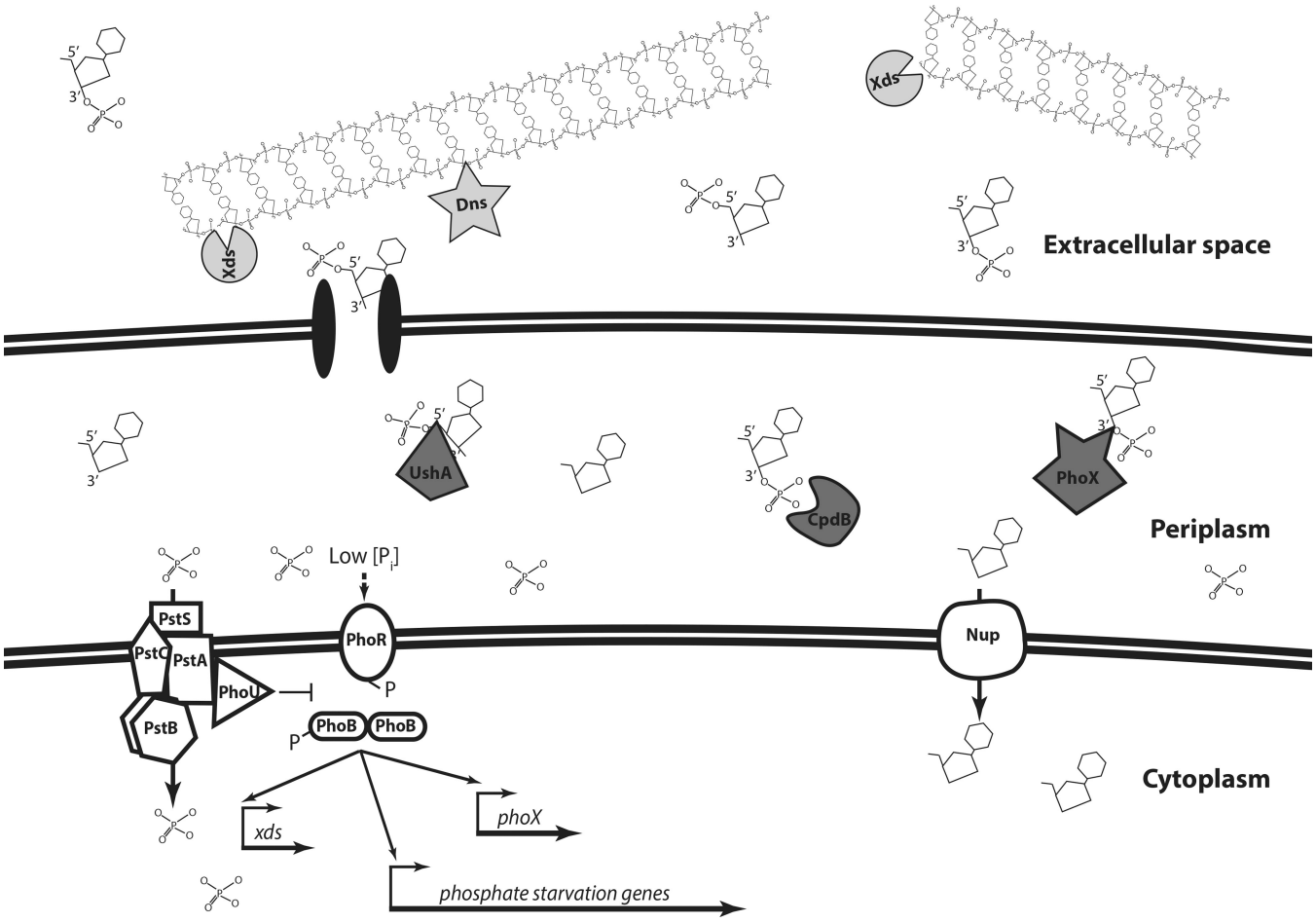

Figure 10. Model for the utilization of eDNA as a source of phosphate in *V. cholerae*

Extracellular DNA is broken down by Xds and Dns in the extracellular space. Dns is an endonuclease in the EndA family of nuclease, and presumably cleaves at the 3' carbon, leaving a 5' phosphate attached to the DNA strand. Alternatively, Xds is an exonuclease and may cleave at the 5' carbon, leaving a 3' phosphate. Once produced, nucleotides can pass across the outer membrane into the periplasm through porins. UshA and CpdB, which we hypothesize as located in the periplasm, remove phosphate groups from 5' and 3'nucleotides, respectively. Additionally, we hypothesize that PhoX contributes to removal of phosphate from 3'dAMP and 3'dCMP. Released phosphate can traverse the inner membrane via the Pst/PhoU system, while nucleosides can pass through the nucleoside transporters (*e.g.* NupC). Low phosphate conditions induce transcription of phosphate starvation genes such as *xds* and *phoX*, in a PhoB-dependent manner. Additionally, CpdB activity is induced under phosphate limiting conditions.

Table 1

Nucleotide growth curve doubling times.

Strain	0.1 mM KH_2PO_4	5'dAMP	5'dGMP	5'dCMP	5'TMP	3'AMP	3'dGMP	3'CMP	3'TMP	No phosphate
Wild type	56 (4)	51 (7)	58 (4)	47 (4)	47 (7)	58 (5)	54 (7)	216 (3)	144 (2)	222 (12)
Δ*phoX*	40 (4)	56 (4)	43 (2)	41 (2)	60 (4)	60 (6)	56 (6)	228 (4)	120 (2)	222 (10)
Δ*ushA*	53 (4)	174 (5)	300 (4)	300 (4)	186 (5)	60 (3)	52 (5)	348 (3)	138 (2)	396 (10)
Δ*cpdB*	59 (5)	42 (5)	50 (2)	52 (2)	45 (5)	181 (3)	132 (6)	222 (3)	174 (2)	186 (8)
ΔVCA0545	39 (4)	32 (4)	44 (4)	45 (4)	38 (4)	49 (4)	52 (4)	216 (4)	ND	198 (4)
ΔVCA0608	66 (3)	53 (8)	50 (2)	49 (2)	51 (8)	59 (6)	47 (6)	180 (2)	126 (2)	204 (10)

Table 2

List of strains used in this study.

Strain	Genotype or Phenotype	Reference
V. cholerae		
Wild type	E7946 El tor Ogawa, HapR+, Ap^R	Laboratory strain
Δ*xds* Δ*dns*	In frame deletion of *xds* and dns	Laboratory strain
Δ*phoX*	In frame deletion of *phoX*	Laboratory strain
Δ*ushA*	In frame deletion of *ushA*, FRT scar	This study
Δ*nupC*	In frame deletion of *nupC*, FRT scar	This study
Δ*cpdB*	In frame deletion of *cpdB*, clean deletion	This study
ΔVCA0545	In frame deletion of VCA0545, FRT scar	This study
ΔVCA0608	In frame deletion of VCA0608, FRT scar	This study
Δ*ushA* Δ*cpdB*	In frame deletion of *ushA* (FRT scar) and *cpdB* (FRT scar)	This study
Δ*ushA* Δ*cpdB* Δ*phoX*	In frame deletion of *ushA* (FRT-Spec-FRT cassette), *cpdB* (FRT scar), and *phoX* (clean deletion)	This study
Δ*ushA* Δ*cpdB* ΔVCA0545	In frame deletion of *ushA* (FRT scar), *cpdB* (FRT scar), and VCA0545 (FRT scar)	This study
Δ*ushA* Δ*cpdB* ΔVCA0608	In frame deletion of *ushA* (FRT scar), *cpdB* (FRT scar), and VCA0608 (FRT scar)	This study
Δ5 phosphatases	In frame deletion of *ushA* (FRT-Spec-FRT cassette), *cpdB* (FRT scar), *phoX* (clean deletion), VCA0545 (FRT scar), and VCA0608 (FRT scar)	This study
Δ*ushA* pMMB67EH	In frame deletion of *ushA* carrying pMMB67EH, Ap^R	This study
Δ*ushA* pMMB67EH-*ushA*	In frame deletion of *ushA* carrying pMMB67EH-*ushA*, Ap^R	This study
Δ*cpdB* pMMB67EH	In frame deletion of *cpdB* carrying pMMB67EH, Ap^R	This study
Δ*cpdB* pMMB67EH-*cpdB*	In frame deletion of *cpdB* carrying pMMB67EH-*cpdB*, Ap^R	This study
E. coli		
DH5αλ*pir*	F- Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* λ::*pir*	Laboratory strain
TG1 pBAD33*kan*	F' [*traD36 proAB*$^+$ *lacI*^{q}*lacZ*ΔM15] *supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*) 5, (rk-, mk-), carrying pBAD33*kan*	Laboratory strain
MFD*pir*	MG1655 RP4-2-Tc::[ΔMu1::*aac*(3)IV-Δ*aphA*-Δ*nic35*-ΔMu2::*zeo*] Δ*dapA*::(*erm*-pir) Δ*recA*	(Ferrieres *et al.*, 2010)
Plasmids		
pDL1098-flp	Temperature-sensitive Flp recombinase delivery vector, Fig. S2, Cm^R	This study; (McDonough et al., 2014)
pBAD33*kan*		Laboratory plasmid
pDL1098	Temperature-sensitive mTn*10* delivery vector, Fig. S1, Cm^R, Sp^R	This study; (McDonough et al., 2014)
pMMB67EH	pMMB67EH IncQ *lacI* ^{q} *bla* (Ap^R) P_{tac} *rrnB*	(Furste *et al.*, 1986)
pMMB67EH-*ushA*	pMMB67EH with the *ushA* ORF cloned into SacI and PstI restriction sites	This study; (McDonough et al., 2014)
pMMB67EH-*cpdB*	pMMB67EH with the *cpdB* ORF cloned into SacI and PstI restriction sites	This study; (McDonough et al., 2014)

Table 3

List of primers used in this study.

Primer use	Primer name	Sequence (5' to 3' orientation)
Arbitrary primed PCR		
	Arb1	(Hava and Camilli 2002)
	olj363	(McDonough *et al* 2014)
	Arb2	(Hava and Camilli 2002)
	olj386	(McDonough *et al* 2014)
qPCR primers		
phoX	VCA0033 qPCR F	CGGTGTCACCATTGTTGAAG
	VCA0033 qPCR R	TGATCCGACGATTACGTTCA
phoB	PhoBqF	AGGGCTATCAGGCGGTTGAG
	PhoBqR	TACCACCAGGCAACATCCAG
cpdB	cpdB qRT F	AGATAAAGCCTCCGATCAAAT
	cpdB qRT R	GATCAAATCACCGTTATCGAC
ushA	ushA qPCR F	GTACCAGAATCAGACCTACAAGA
	ushA qPCR R	GGATTATCAAATTCGTGGTTAC
Strain construction		
Δ*ushA*	ushA FRT F1	TCACATCGAGTTAGCACGTCTG
	ushA FRT R1	GTCGACGGATCCCCGGAATCATTGTCATACCTTTGAACTGATG
	ushA FRT F2	GAAGCAGCTCCAGCCTACATAATAAGGTTTGACTCGCAAAGTTG
	ushA FRT R2	AGAGGTTACAGGAGTGCGTCAG
	ushA R0	CTTTGCGCACTTTGATGAAT
Δ*nupC*	nupC FRT F1	TACACTGAGCTGCAACGCATTG
	nupC FRT R1	GTCGACGGATCCCCGGAATCAAATTGTGAGTAGAACAGGAAAGG
	nupC FRT F2	GAAGCAGCTCCAGCCTACATGATCACAGATTGATGGATTGAG
	nupC FRT R2	GTTAAGGGTAATAGTGCCTTCAGC
	nupC R0	CCGACTAAAAACTCCACCTGA
ΔcpdB	cpdB F1	TCTCGGTCTCTCCCTGTAAATG
	cpdB R1	TGTAGGCTGGAGCTGCTTCTTCACTCATAACCAAATTGTGATGTG
	cpdB F2	GAAGCAGCTCCAGCCTACATAAGCACCGATAATGCCCCTATTG
	cpdB R2	CCTCATAGAAAAGAAAACAGCC
	cpdB R0	CCTCATAGAAAAGAAAACAGCC
ΔVCA0545	VCA0545 F1	GGTGTGAAAAGTACCAAGGGA
	VCA0545 R1	TGTAGGCTGGAGCTGCTTCGGCATACGTCTTCTCTCTTTTC
	VCA0545 F2	GAAGCAGCTCCAGCCTACATAAAACGGATATCTCTTTGCCTT
	VCA0545 R2	CAGTTCCAAAGCTCACTCC
	VCA05454 R0	CGTTCGGCTTACCATTTTTCT
ΔVCA0608	VCA0608 F1	TTTCGTTGGATGTTGACACTG
	VCA0608 R1	TGTAGGCTGGAGCTGCTTCTTCATGATGATCTCCTTAAAATCAG

Primer use	Primer name	Sequence (5' to 3' orientation)
	VCA0608 F2	GAAGCAGCTCCAGCCTACATAATGCCATGAATAAGCGAGG
	VCA0608 R2	TCATCACCTCTTTCTATTCACC
	VCA0608 R0	AGAGCTAGAGAAACTGGAAGAA
FRT scar screening	ABD725	GAAGCAGCTCCAGCCTACA
pMMB67EH-*ushA*	pMMB-ushA F	ATCGGAGCTCATGAAACAAGGCCTCATTCTA
	pMMB-ushA R	ATCGCTGCAGTTAACGATAAACAATCTCGCCCG
pMMB67EH-*cpdB*	pMMB-cpdB F	ATCGGAGCTCGTGAAACCTTTGTTTCATCGA
	pMMB-cpdB R	ATCGCTGCAGTTATTTTTGTAAGTCGATGCGAT