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Phosphite utilization by the marine picocyanobacterium *Prochlorococcus* MIT9301

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Summary

Primary productivity in the ocean's oligotrophic regions is often limited by phosphorus (P) availability. In low phosphate environments, the prevalence of many genes involved in P acquisition is elevated, suggesting that the ability to effectively access diverse P sources is advantageous for organisms inhabiting these regions. *Prochlorococcus,* the numerically dominant primary producer in the oligotrophic ocean, encodes high-affinity P transporters, P regulatory proteins and enzymes for organic phosphate utilization, but its ability to use reduced P compounds has not been previously demonstrated. Because *Prochlorococcus* strain MIT9301 encodes genes similar to *phnY* and *phnZ*, which constitute a novel marine bacterial 2-aminoethylphosphonate (2-AEPn) utilization pathway, it has been suggested that this organism might use 2-AEPn as an alternative P source. We show here that although MIT9301 was unable to use 2-AEPn as a sole P source under standard culture conditions, it was able to use phosphite. Phosphite utilization by MIT9301 appears to be mediated by an NAD-dependent phosphite dehydrogenase encoded by *ptxD*. We show that phosphite utilization genes are present in diverse marine microbes and that their abundance is higher in low-P waters. These results strongly suggest that phosphite represents a previously unrecognized component of the marine P cycle.

Introduction

Phosphorus (P) is an essential element for living organisms. In its most oxidized state (valence +5), it is found as phosphate esters in many biomolecules, including nucleic acids, phospholipids and phosphoproteins, and plays a central role in many metabolic pathways including energy transfer reactions involving nucleotide cofactors. It has become apparent, however, that P+5 is not the only P species available to living organisms, as pathways for metabolism of P+3 compounds (phosphonates and phosphite) and even P+1 hypophosphite have been recently described (reviewed in Quinn *et al.*, 2007; White and Metcalf, 2007; Metcalf and van der Donk, 2009). For example, phosphite and hypophosphite can be used as the sole P source by *Pseudomonas stutzeri* WM88 and *Alcaligenes faecalis* WM2072. In these organisms, hypophosphite oxidation is carried out by a 2-oxoglutarate-dependent dioxygenase encoded by the *htxA* gene, while phosphite oxidation to phosphate is catalysed by the NAD-dependent phosphite dehydrogenase, PtxD (Metcalf and Wolfe, 1998; White and Metcalf, 2004; Wilson and Metcalf, 2005). Phosphite oxidation is also carried out by *Desulfotignum phosphitoxidans* which can remarkably not only use phosphite as the sole P source, but also as the sole electron donor for sulfate to sulfite reduction during anaerobic respiration (Schink *et al.*, 2002; Simeonova *et al.*, 2010).

Metabolic pathways for the utilization of phosphonate [reduced organic P(+3) compounds characterized by a direct P–C bond], have also been described (Quinn *et al.*, 2007; White and Metcalf, 2007). These include broad-specificity pathways such as the C–P lyase enzyme complex (Metcalf and Wanner, 1991; 1993a,b), as well as substrate-specific pathways. Well-characterized substrate-specific C–P hydrolases include phosphonoacetate hydrolase (McMullan and Quinn, 1994), phosphonopyruvate hydrolase (Ternan *et al.*, 1998; 2000; Ternan and Quinn, 1998a) and phosphonoacetaldehyde hydrolase (also known as phosphonatase) (Baker *et al.*, 1998; Ternan and Quinn, 1998b). Microbial phosphonate biosynthetic pathways have also been described and include pathways for the biosynthesis of important natural products such as the antibiotic fosfomycin, and the herbicide phosphinothricin (reviewed in Metcalf and van der Donk, 2009).

The use of reduced P compounds as a source of P by marine organisms is of particular interest because these compounds represent a significant fraction of the P pool in the oceans, and P availability limits the growth of

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microorganisms in many marine environments. Indeed, it has long been known that many marine protozoans actually harbour a significant portion of their P as phosphonates, including 2-AEPn and phosphonoalanine, among others (Quin, 1965; Horiguchi, 1984; 1991). More recently, the cyanobacterium *Trichodesmium erythraeum* was reported to be a potentially important source of phosphonates in oligotrophic systems (Dyhrman *et al*., 2009). In addition, phosphonates represent a significant proportion of the dissolved organic P (DOP) in all marine environments analysed. Although the exact chemical structures are not known, ^{31}P-NMR studies have revealed that phosphonates account for approximately 25% of the high-molecular-weight DOP across sites and depths (Clark *et al*., 1999; Kolowith *et al*., 2001). Further, relative to phosphate esters, phosphonates are preferentially removed from sinking particles, again suggesting that these compounds are an important source of bioavailable P in marine ecosystems (Benitez-Nelson *et al*., 2004). Not surprisingly, considerable evidence for phosphonate utilization by marine microbes has accumulated in recent years starting with the report that *T. erythraeum* IMS101 has a C–P lyase gene cluster that is expressed under low-P conditions (Dyhrman *et al*., 2006). Since then, several diverse marine strains have been shown to grow with phosphonates as the sole P source, including the cyanobacterium *T. erythraeum* IMS101, *Ruegeria pomeyori* DSS-3, *Planctomyces maris* DSM8797, *Photobacterium profundum* S14 and coral-associated *Vibrionacea* (Gilbert *et al*., 2009; Martinez *et al*., 2010; White *et al*., 2010). Furthermore, analyses of data collected in metagenomic surveys have found a high incidence and diversity of phosphonate degradation genes in the marine environment, particularly in low-P surface waters of the Sargasso and Mediterranean Seas, and below the photic zone (Quinn *et al*., 2007; Karl *et al*., 2008; Gilbert *et al*., 2009; Coleman and Chisholm, 2010; Feingersch *et al*., 2010; Martinez *et al*., 2010; Luo *et al*., 2011).

Recently, a novel two-gene phosphonate degradation pathway was discovered in a marine genomic fragment by functional gene complementation (Martinez *et al*., 2010). A putative 2-oxoglutarate dioxygenase, *phnY*, and a possible phosphohydrolase, *phnZ*, were sufficient to allow utilization of 2-AEPn as the sole P source in *Escherichia coli*. Orthologues of these genes were identified in the genomes of several marine bacterial species, including two strains of the picocyanobacterium *Prochlorococcus,* MIT9301 and MIT9303, suggesting that they too might be able to use 2-AEPn. Interestingly, the frequency of the *Prochlorococcus phnY* and *phnZ* genes was significantly higher in the P-depleted surface waters of the Sargasso Sea compared with the North Pacific Gyre, and *phnY* and *phnZ* expression was induced in cultures of MIT9301 following P depletion (Coleman and Chisholm, 2010; Martinez *et al*., 2010). Given the importance of *Prochlorococcus* in the oligotrophic environments of low- to mid-latitude oceans where it can account for ~30% of primary productivity (Goericke and Welschmeyer, 1993), the utilization of reduced P compounds by this organism could have important ecological implications. Phosphonate utilization by *Prochlorococcus* strains had been previously hypothesized based on the presence of a separate putative phosphonate ABC transporter gene cluster in the genomes of all sequenced *Prochlorococcus* strains, but previous attempts to demonstrate phosphonate utilization in this microbial group have been unsuccessful (Moore *et al*., 2005; Martiny *et al*., 2006).

Here we investigate the ability of *Prochlorococcus* MIT9301 to grow using reduced P compounds, including phosphite and 2-AEPn. Although we saw no evidence of 2-AEPn utilization, we showed that MIT9301 can in fact use phosphite as the sole P source in culture, mediated by a phosphite dehydrogenase encoded by a *ptx* gene cluster similar to that of other phosphite utilizing bacteria. This cluster is also present in other marine organisms suggesting that phosphite might be an important, previously unrecognized component of the marine P cycle.

Results

A putative phosphonate utilization gene cluster in Prochlorococcus *MIT9301 and MIT9303 contains phosphite utilization genes*

As reviewed above, we have recently shown that two genes constituting a new phosphonate utilization pathway found in a marine metagenomic fragment, *phnY* and *phnZ*, allow utilization of 2-AEPn as a sole P source when expressed in *E. coli* (Martinez *et al*., 2010). Similarity searches revealed that the genomes of *Prochlorococcus* strains MIT9301 and MIT9303 encode *phnY* and *phnZ* homologues (Fig. 1A), and *phnY* and *phnZ* have been shown to be expressed under P starvation in MIT9301 cultures suggesting that these strains might be capable of utilizing 2-AEPn as a P source (Coleman and Chisholm, 2010; Martinez *et al*., 2010). *phnY* encodes a predicted 2-oxoglutarate dioxygenase, while *phnZ* encodes a predicted protein of the HD phosphohydrolase family. Both genes, in a similar arrangement, are also found in MIT9303 (Fig. 1A).

The gene directly upstream of *phnY* and *phnZ* in the MIT9301 genome (Fig. 1A) (Kettler *et al*., 2007), which is absent in MIT9303, encodes a hypothetical protein of unknown function of the *N*-acetyltransferase superfamily. Adjacent to this gene, and transcribed in the same direction as *phnYZ*, is a four-gene cluster in which three loci are predicted to encode the ATPase, periplasmic and permease components of a putative phosphonate ABC trans-

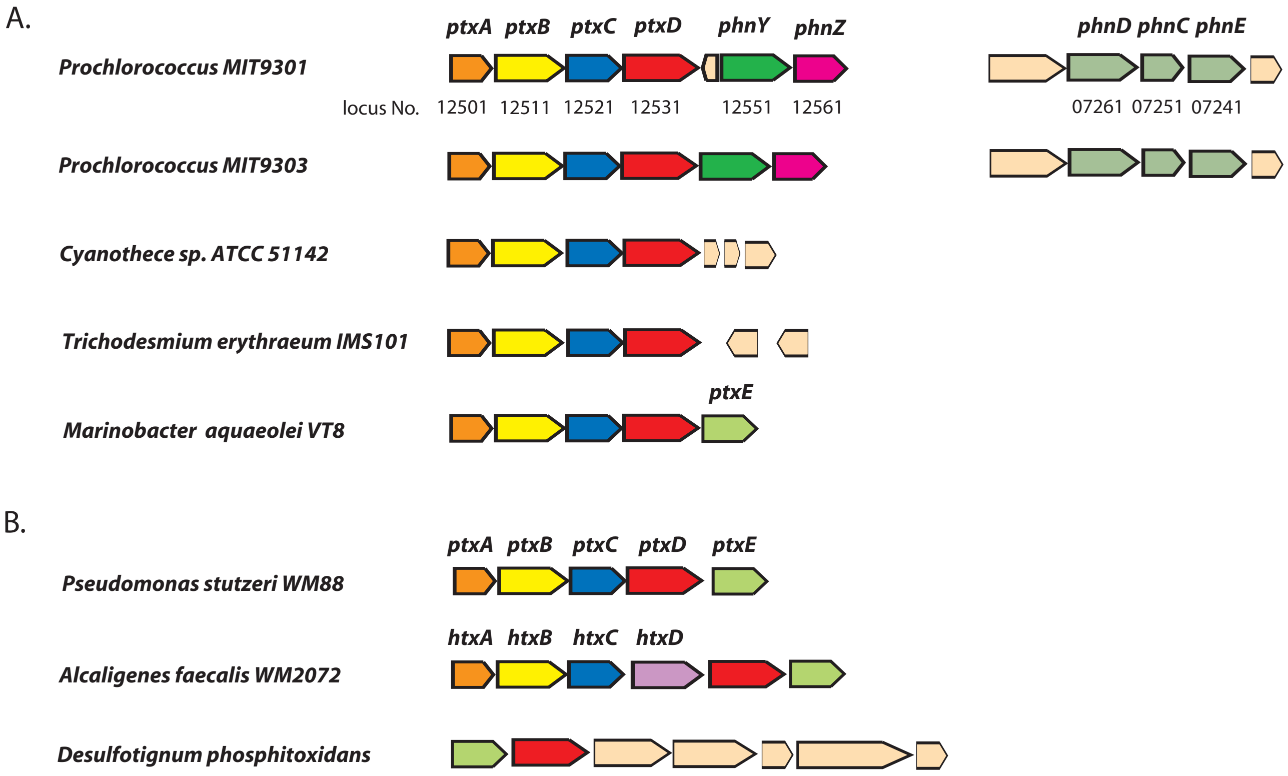

Fig. 1. A. Phosphite and phosphonate utilization genes in *Prochlorococcus* MIT9301, *Prochlorococcus* MIT9303 and other marine strains. The clusters on the left include the three components of the putative phosphite ABC transporter (*ptxA, ptxB* and *ptxC*), *ptxD* which encodes phosphite dehydrogenase, and *phnY* and *phnZ*, which are similar to genes encoding a 2-AEPn utilization pathway in other marine bacteria, and here are present only in MIT9301 and MIT9303. A second cluster (right), present in all *Prochlorococcus* and *Synechococcus* genomes, includes *phnCDE*, encoding a putative phosphonate ABC transporter of unknown function. Locus numbers are indicated for MIT9301.
B. Gene arrangement in three bacteria for which the PtxD system has been characterized genetically and/or biochemically (*Pseudomonas stutzeri* WM88, *Alcaligenes faecalis* WM2072 and *Desulfotignum phosphitoxidans*).

porter system. Expression of the periplasmic and permease component genes has been shown to increase under P-starvation conditions (Coleman and Chisholm, 2010). The fourth locus in the cluster was annotated as a putative dehydrogenase because of its similarity to COG1052 (lactate dehydrogenase and related dehydrogenases) (Kettler *et al.*, 2007). This four-gene cluster, also present in MIT9303 adjacent to *phnY* and *phnZ*, is absent from all other *Prochlorococcus* reference genomes and it is distinct from a separate *phnCDE* cluster encoding a putative phosphonate ABC transporter that is present in all *Prochlorococcus* and *Synechococcus* genomes (Fig. 1A). In order to avoid confusion and to better reflect the new physiological information described here, we will herein refer to the four-gene cluster adjacent to *phnYZ* in MIT9301 and MIT9303 as *ptxA, ptxB, ptxC* and *ptxD*. Evidence supporting the re-annotation is presented below.

To shed light on the putative roles of these four genes in reduced P utilization, we compared their predicted amino acid sequences to peptides in the NCBI non-redundant database (Table S1). The predicted protein encoded by *ptxD* (locus P9301_12531) is highly similar to the well-characterized NAD-dependent phosphite dehydrogenases, PtxD, from *A. faecalis* WM2072, *P. stutzeri* WM88, *Xanthobacter flavus* WM2814 and *D. phosphitoxidans* (expectation values of 5×10^{-86}, 1×10^{-83}, 2×10^{-73} and 3×10^{-54} respectively). PtxD allows these organisms to grow with phosphite as the sole P source (Metcalf and Wolfe, 1998; Wilson and Metcalf, 2005; Wilson, 2006; Simeonova *et al.*, 2010). An amino acid alignment between the predicted protein encoded by *ptxD* (P9301_12531) and those of the four known PtxD proteins from other organisms (Fig. S1) shows extensive sequence similarity throughout the length of the protein. More importantly, the characteristic Rossmann fold motif (G-X-G-X$_2$-G-X$_{17}$-D) for NAD cofactor binding (Rossmann *et al.*, 1974; Wierenga *et al.*, 1985; Woodyer *et al.*, 2003), and all the amino acid residues shown to be involved in substrate binding or catalysis in *P. stutzeri* PtxD (Woodyer *et al.*, 2003; 2005; Relyea and van der Donk, 2005; Fogle and van der Donk, 2007), are conserved. These results strongly suggest that *ptxD* (P9301_12531) encodes an NAD-dependent phosphite dehydrogenase, hence its re-annotation.

The *ptxD* assignation for P9301_12531 is further supported by phylogenetic analysis. Figure 2 shows that *Prochlorococcus* MIT9301 and MIT9303 PtxD sequences form a well-supported cluster with all four known PtxD proteins, to the exclusion of different functional types of enzymes belonging to the D-hydroxyacid family of proteins, included here as an outgroup. Interestingly, *ptxD* homologues are also found in other species of cyanobacteria as well as in representatives of the α-, β- and γ-*Proteobacteria* (Fig. 2 and Table S1). Several marine strains, including *Cyanothece* sp., *T. erythraeum*, *Nodularia spumigea* and *Marinobacter* sp. are included in this group, suggesting that bacterial phosphite utilization might be widespread in the ocean environment.

As discussed above, the *Prochlorococcus* MIT9301 *ptxD* gene resides in a predicted operon with genes encoding an ABC type transporter (Fig. 1A). That is also the case for most of the other *ptxD*-containing genomes (Fig. 1B). The predicted protein sequences encoded by these three genes are more similar to those of the ATPase (PtxA), periplasmic binding protein (PtxB) and permease (PtxC) components of the phosphite transporter of *P. stutzeri* WM88 (Table S1), required for phosphite utilization by this organism (Metcalf and Wolfe, 1998), than to PhnCDE, the components of the second putative phosphonate ABC transporter predicted in most sequenced *Prochlorococcus* and *Synechococcus* strains, including *Prochlorococcus* strains MIT9301 and MIT9303 (Palenik *et al.*, 2003; Rocap *et al.*, 2003; Su *et al.*, 2003; Kettler *et al.*, 2007). The function of this putative orphan PhnCDE transporter in *Prochlorococcus* is unknown; it is not linked to any known phosphonate utilization gene, and its expression appears to be constitutive *in situ* and unresponsive to P starvation in culture (Martiny *et al.*, 2006; Ilikchyan *et al.*, 2009; 2010). Phylogenetic analysis of the periplasmic phosphite-binding proteins (Fig. 3) reveals that the periplasmic binding protein associated with PtxD in MIT9301 forms a coherent cluster with the phosphite-binding protein PtxB of *P. stutzeri*, and of other bacteria. This lineage is distinct from that of the phosphonate-binding protein PhnD associated with C–P lyase gene clusters, as well as from the cluster containing the orphan *Prochlorococcus* PhnD discussed above.

Based on the above sequence similarities, phylogenetic analyses and genetic linkage data, we propose that loci *ptxA, ptxB* and *ptxC* in MIT9301 and MIT9303 encode a phosphite-specific ABC transporter that is associated with the *ptxD* phosphite dehydrogenase gene. This *ptxABCD* operon appears to be widespread among diverse sequenced bacteria, and is so far linked to the *phnYZ* operon only in *Prochlorococcus* strains MIT9301 and MIT9303 (Fig. 1). This finding raises the possibility that the *ptxABCD* and *phnYZ* genes may have evolved independently and were joined by a lateral gene transfer event.

Prochlorococcus *MIT9301 can use phosphite as the sole P source*

The presence of the putative *ptxABCD* (phosphite utilization) and *phnYZ* (2-AEPn utilization) pathways raises the question of whether *Prochlorococcus* MIT9301 and MIT9303 can use phosphite and 2-AEPn as a P source. To test this hypothesis we analysed the ability of three axenic *Prochlorococcus* strains to grow with phosphate, phosphite, or 2-AEPn as P source (we did not test MIT9303 because an axenic strain was not available). Among the three strains, only MIT9301 has the *ptxABCD*–*phnYZ* cluster (Fig. 1), while all three have the *phnCDE* genes encoding the putative phosphonate transporter. No additional known phosphonate utilization genes are found in any of the strains. Under our culture conditions, none of strains was able to grow using 2-AEPn as the sole P source (Fig. 4). Two other related phosphonate compounds tested, ethylphosphonate and phosphonoalanine, also failed to support growth (data not shown). This result was unexpected for MIT9301 because its *phnYZ* genes are similar to those identified previously in a marine metagenomic fragment that allowed growth on 2-AEPn in *E. coli* (Martinez *et al.*, 2010), and transcript levels of these genes were increased during P starvation of *Prochlorococcus* MIT9301 in culture (Coleman and Chisholm, 2010).

In contrast, Fig. 4 shows that of three axenic *Prochlorococcus* strains (MED4, MIT9301 and MIT9313), only MIT9301, the only one of the three that contains the *ptxABCD* cluster, was capable of growth using phosphite. Under our experimental conditions, MIT9301 consistently grew more slowly with phosphite than with phosphate, regardless of which P source had been used to grow culture inoculums. The average growth rates across experiments were $\mu = 0.7 \pm 0.11$ day^{-1} for phosphate and $\mu = 0.18 \pm 0.06$ day^{-1} for phosphite. Further, exponential growth rates using phosphite were similar over a 100-fold range of phosphite concentrations, whereas growth yields under limiting phosphite concentrations were reduced and were similar to those achieved with the equivalent concentration of phosphate. In addition, media with equimolar amounts of phosphate and phosphite yielded the same results as that with phosphate only, demonstrating that phosphite is not toxic to the cells (Fig. S2). Control experiments indicated that phosphite was stable in the growth medium for the duration of the experiment, i.e. phosphate was undetectable in light-exposed phosphite-

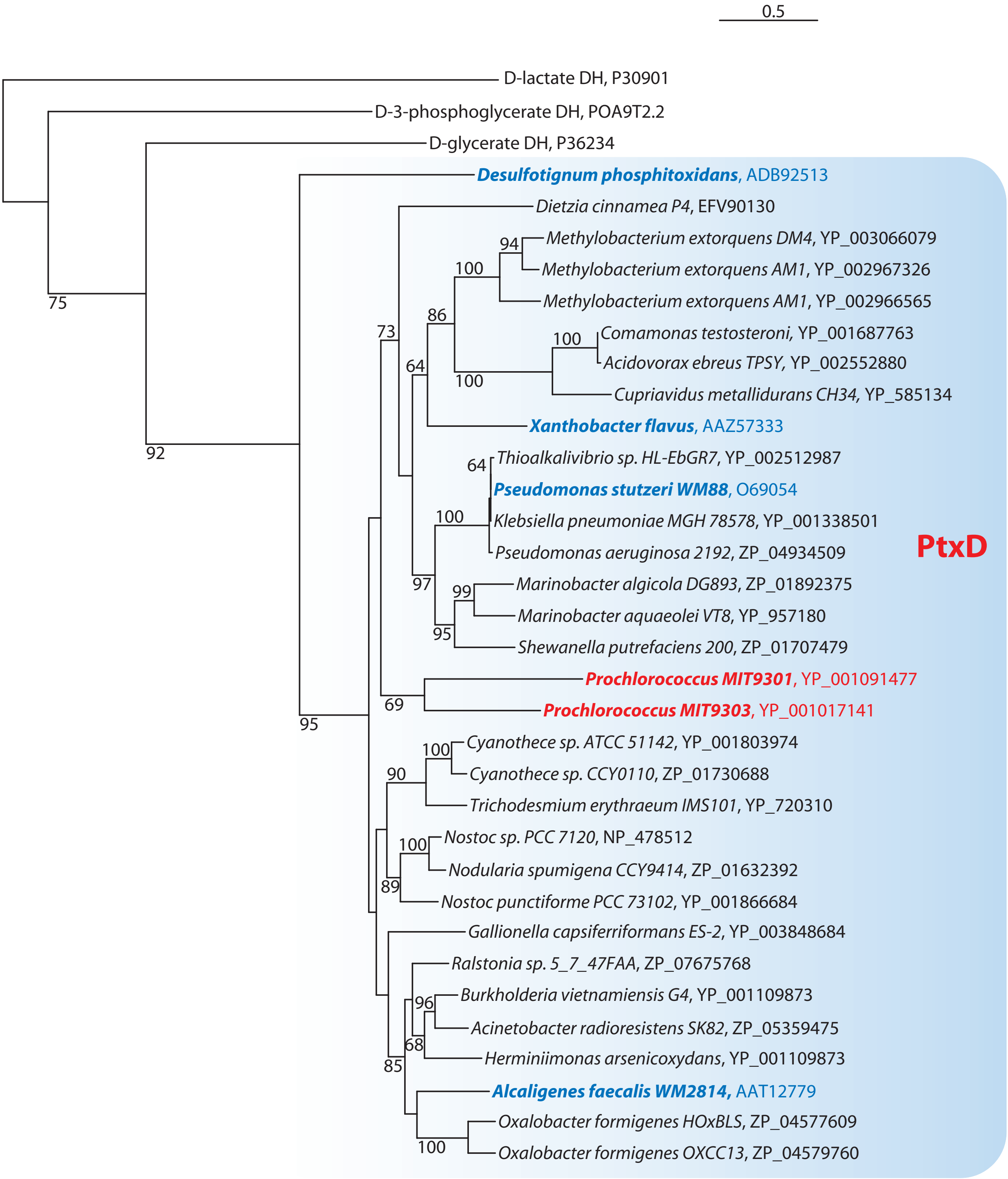

Fig. 2. Maximum likelihood phylogenetic tree for the NAD-dependent phosphite dehydrogenase PtxD. The following well-characterized members of the D-hydroxyacid dehydrogenase family with different substrate specificity were included as outgroups: D-lactate dehydrogenase (*Lactobacillus helveticus*, P30901), D-3-phosphoglycerate dehydrogenase (*Escherichia coli*, POA9T2.2) and D-glycerate dehydrogenase (*Hypomicrobium methylovorum*, P36234). *Prochlorococcus* MIT9301 and MIT9303 are highlighted in red. Organisms for which there is biochemical and/or genetic evidence for PtxD function are highlighted in blue. The PtxD phylogenetic cluster is highlighted with a blue box. Bootstrap values > 50 are included for the corresponding nodes. The scale bar is equal to 0.5 changes per amino acid residue.

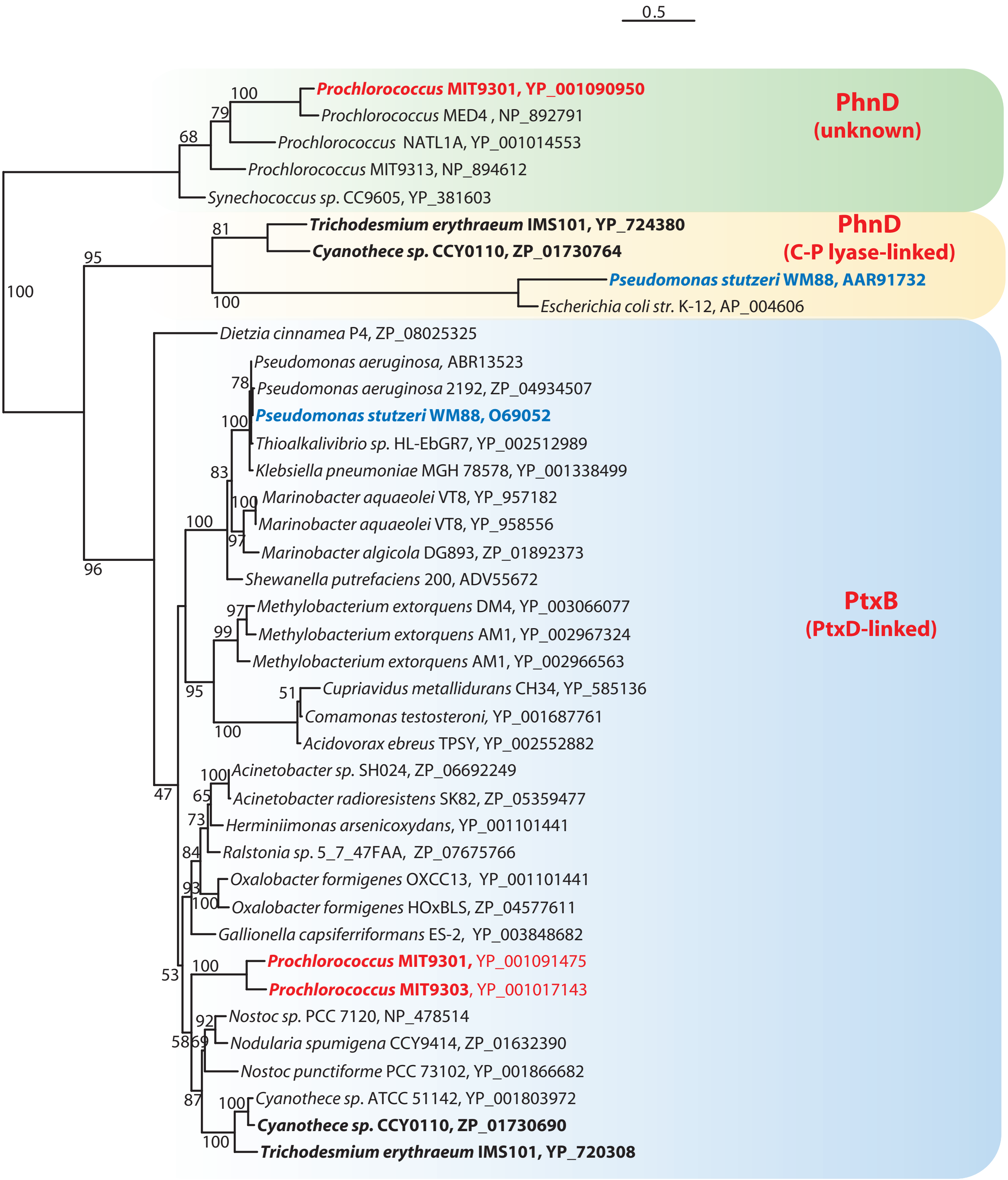

Fig. 3. Maximum likelihood phylogenetic tree for the phosphite-binding periplasmic protein PtxB. Other predicted phosphonate-binding proteins with lower similarity to MIT9301 PtxB were included in the analyses. Bootstrap values > 50 are included for the corresponding nodes. The three major phylogenetic clusters are highlighted with coloured boxes as follows: blue, predicted PtxB proteins encoded by genes linked to the *ptxD* gene encoding phosphite dehydrogenase; yellow, PhnD phosphonate-binding proteins associated with C–P lyase clusters; and green, putative PhnD phosphonate-binding protein in *Prochlorococcus* and *Synechococcus*.

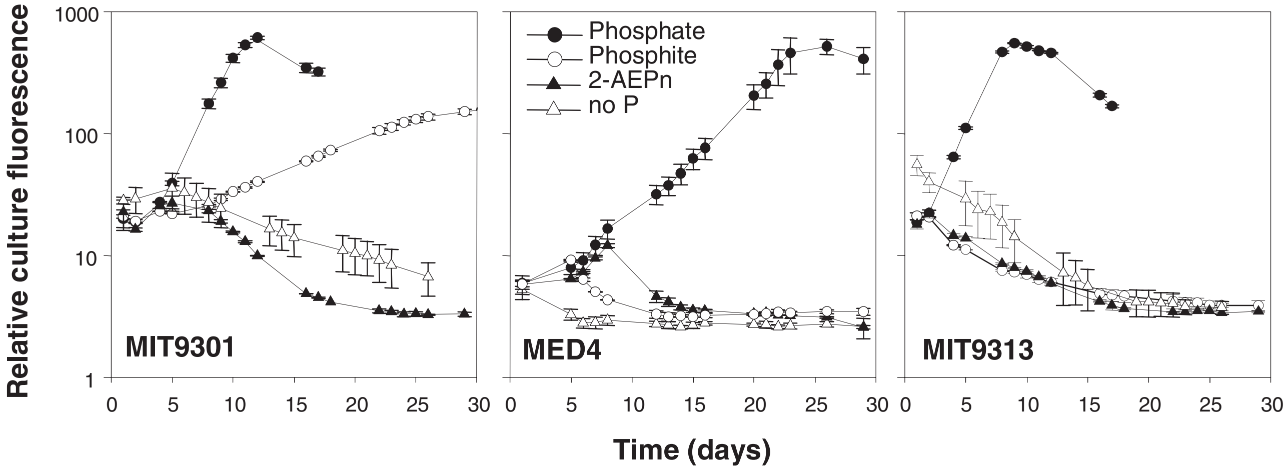

Fig. 4. *Prochlorococcus* strain MIT9301 can use phosphite as the sole P source. Axenic strains of *Prochlorococcus* were grown to mid-logarithmic phase in phosphate-containing Pro99 medium, then washed on filters and resuspended in medium containing 50 μM P source as indicated. —●—: phosphate; —△—: No P; —▲—: 2-aminoethylphosphonate; —○—: phosphite. Error bars represent the standard deviation of the mean relative fluorescence of duplicate cultures. MIT9303 strain was not tested because an axenic strain was not available.

containing medium, and MED4, which lacks the *ptxABCD* cluster, could not grow on the aged phosphite medium without the addition of phosphate (data not shown). To our knowledge, this is the first report of phosphite utilization by a marine bacterium.

MIT9301 ptxD *complements an* E. coli *phosphite utilization mutant* in vivo

As there is currently no genetic system available for *Prochlorococcus*, we cloned and expressed the MIT9301 *ptxD* gene in *E. coli* BW16787 (Lee *et al*., 1992; Jiang *et al*., 1995) in order to establish a direct link between this gene and the ability of MIT9301 to use phosphite as a P source. *Escherichia coli* strain BW16787 cannot use either phosphite or phosphonates because of a deletion of the *phnH–P* genes necessary for C–P lyase activity (Metcalf and Wanner, 1991) and a mutation in *phoA* (alkaline phosphatase), which is important because *E. coli*'s alkaline phosphatase can oxidize phosphite to phosphate (Yang and Metcalf, 2004). However, because the corresponding phosphonate ABC transporter genes *phnCDE* are intact in this strain, it is a useful tool for validating heterologous genes encoding reduced P utilization enzymes by means of gene complementation (Lee *et al*., 1992; Jiang *et al*., 1995; Martinez *et al*., 2010).

We cloned the MIT9301 *ptxD* gene into a high-copy-number plasmid under control of the *E. coli lac* promoter, and showed that BW16787 harbouring the plasmid containing MIT9301 *ptxD* in the forward orientation was capable of utilizing phosphite as the sole P source whereas BW16787 harbouring the plasmid with *ptxD* in the reverse orientation or an empty plasmid, could not (Fig. 5A). We also analysed the specificity of the cloned *ptxD* gene with regard to utilization of phosphonate compounds, and found that MIT9301 *ptxD* did not allow *E. coli* BW16787 to use any of the phosphonate compounds tested (Fig. 5B). Similar narrow substrate specificity has been described for the other known NAD-dependent phosphite dehydrogenases (Garcia Costas *et al*., 2001; Wilson, 2006).

MIT9301 phnYZ *does not complement phosphonate utilization in* E. coli

We also tested the ability of the MIT9301 *phnY* and *phnZ* genes to complement BW16787 for 2-AEPn utilization, an approach used successfully for identifying the original *phnYZ* genes in a metagenomic library (Martinez *et al*., 2010). In this case, however, the cloned *phnYZ* genes were not capable of supporting BW16787 growth with 2-AEPn or with any of the other reduced P compounds tested (Fig. 5B).

Abundance and expression of phosphite and phosphonate genes in metagenomic and metatranscriptomic databases

Prochlorococcus MIT9301 and MIT9303 were isolated from 90 and 100 m Sargasso Sea water samples (Moore *et al*., 1998; Rocap *et al*., 2002). In order to evaluate the significance of the phosphite and phosphonate utilization genes in wild *Prochlorococcus* populations, we analysed their abundance in metagenomic and metatranscriptomic libraries. Data from depth profiles from the BATS station near Bermuda in the Sargasso Sea and from Station ALOHA in the North Pacific Gyre were examined. These two systems are characterized by vastly different phos-

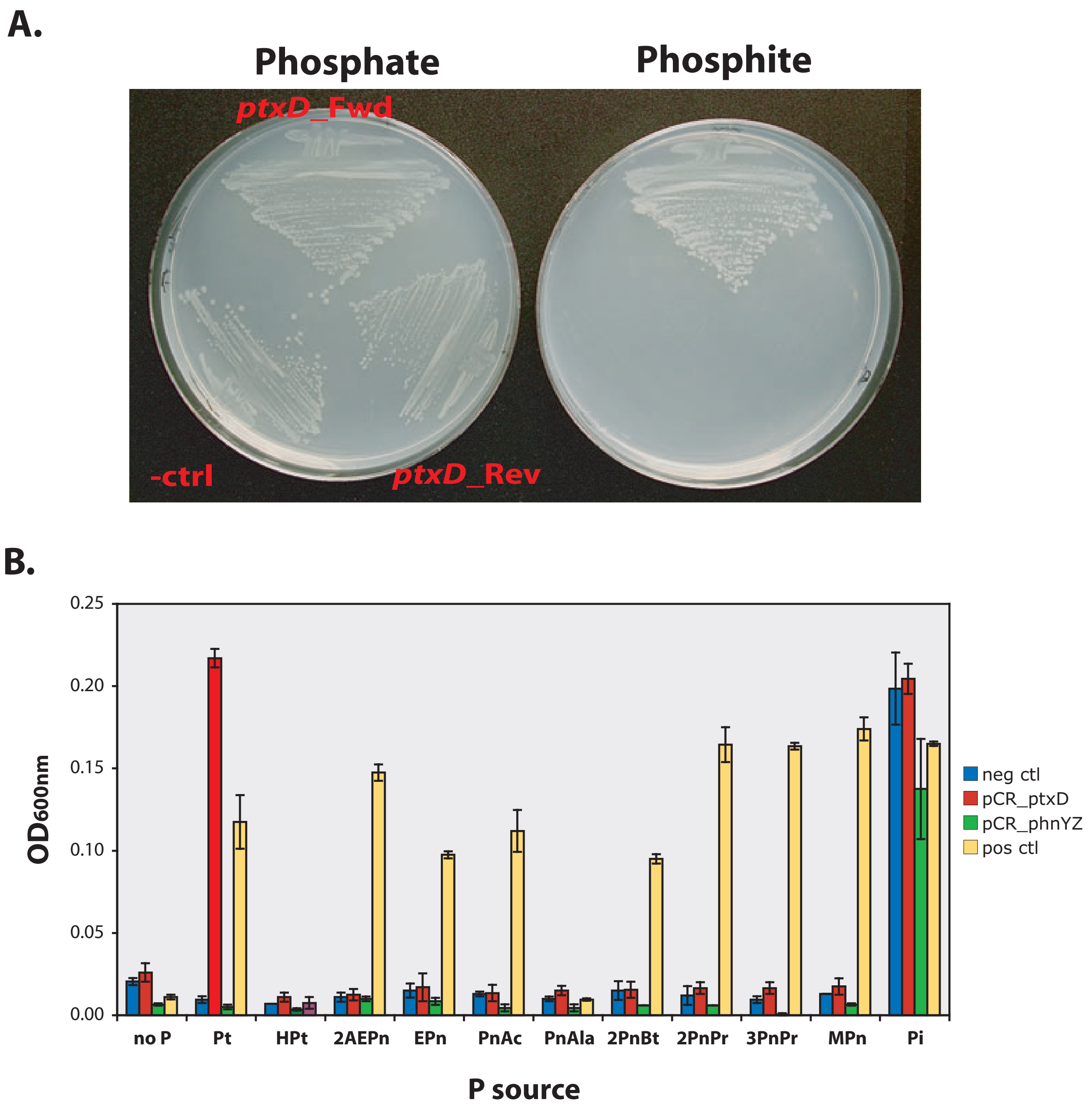

Fig. 5. *Prochlorococcus* MIT9301 *ptxD* allows *E. coli* to use phosphite as the sole P source.

A. The complementation phenotype of plasmids containing *ptxD* in the forward orientation with respect to the P*lac* promoter (pCR-*ptxD*_Fwd), *ptxD* in the reverse orientation (pCR_*ptxD*_Rev) or the empty cloning vector was tested in *E. coli* strain BW16787 (*phoA*, Δ*phnH–P*). Only strains harbouring *Prochlorococcus* MIT9301 *ptxD* in the forward orientation can grow on phosphite as the only P source.

B. Reduced P specificity assay. BW16787 harbouring pCR-ptxD_Fwd, pCR-phnYZ_Fwd, or the empty plasmid control was tested in liquid cultures for the ability to grow on 0.2 mM phosphate (Pi), phosphite (Pt), hypophosphite (HPt), 2-aminoethylphosphonate (2AEPn), ethylphosphonate (EPn), phosphonoacetate (PnAc), phosphonoalanine (PnAla), 2-phosphonobutyrate (2PnBt), 2-phosphonopropionate (2PnPr), 3-phosphonopropionate (3PnPr), methylphosphonate (MPn). BW18812 (Phn$^+$) containing the empty vector was the positive control. Strains containing plasmids with *ptxD* or *phnYZ* in the reverse orientation did not allow cells to use any reduced P source tested (data not shown).

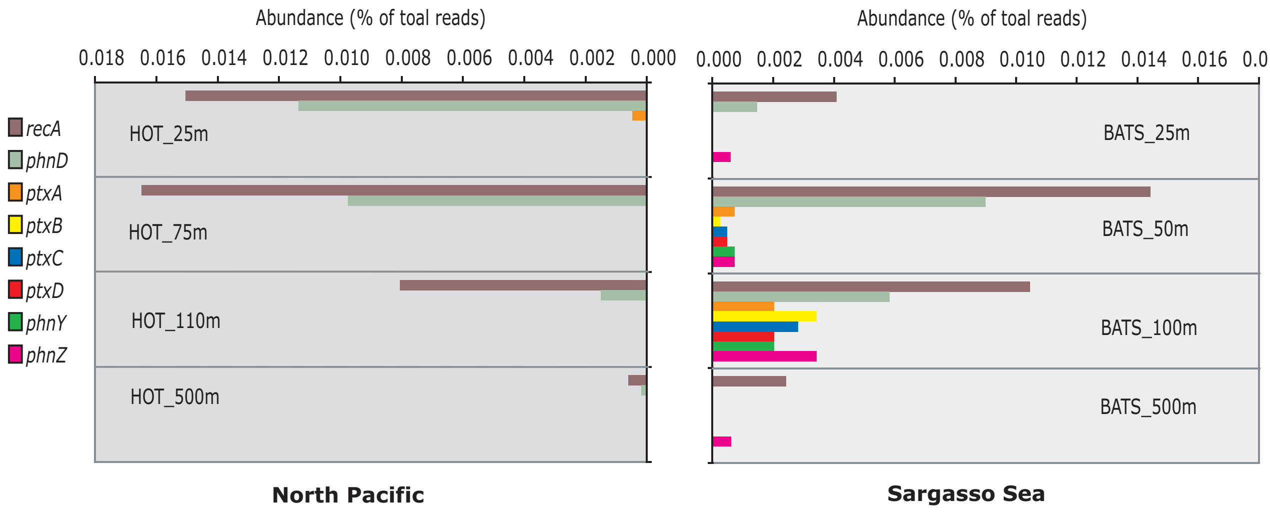

Fig. 6. *Prochlorococcus ptxABCD* and *phnYZ* gene abundance in metagenomic data sets from the North Pacific (HOT186) and Sargasso Sea (BATS216). Gene abundance is expressed as a percentage of the total number of reads in each library. Only reads that match the query sequence (MIT9301 genes) with bit > 50, and % id > 85% were considered significant. *phnD* and *recA* are shown for comparison.

phate concentrations, with concentrations in the Sargasso Sea (0.2–1 nM) more than one order of magnitude lower than those of the North Pacific Gyre (Wu *et al*., 2000). In previous analyses we found significantly higher abundance of phosphonate utilization genes in the genomic DNA of surface samples of the Sargasso Sea, not only of genes *phnY* and *phnZ*, but also genes of the C–P lyase and phosphonatase pathways, as well as other P acquisition genes (Coleman and Chisholm, 2010; Martinez *et al*., 2010). For this study we expanded on the previous work by analysing genomic DNA, and paired cDNA libraries when appropriate, from four depths in each location (the top mixed layer, just below the mixed layer, the deep chlorophyll maximum and the mesopelagic zone), with a particular focus on *Prochlorococcus* phosphite utilization sequences. Available nutrient data for both depth profiles are presented in Fig. S3.

For the purpose of this study, high significance cut-off values (bit score above 50 and at least 85% identity) were chosen to limit our analyses of pyrosequencing data to *Prochlorococcus* sequences. Reads encoding putative proteins with high similarity to MIT9301 PtxABCD and PhnYZ were only found almost exclusively in the 50 and 100 m samples of the Sargasso Sea (Fig. 6). The highest abundance, expressed as a percentage of the total reads in the library, was found in the 100 m genomic DNA sample (0.0020–0.0034, compared with 0.0104 for the single-copy reference gene *recA*). In contrast, significant matches to *recA* or to the putative *phnD* periplasmic-binding protein found in most *Prochlorococcus* reference genomes were detected at significant levels in all photic zone samples from both sites. Using *recA* as a single-copy gene reference, the percentage of cells in the natural *Prochlorococcus* population containing the *ptxABCD* and *phnYZ* genes is estimated to be 2–9% at 50 m and 22–59% at 100 m (Table S2). The high abundance of these genes in the 100 m sample of the Sargasso Sea is consistent with their presence in MIT9301 and MIT9303, two of the three fully sequenced isolates from 90–100 m Sargasso Sea waters.

We also analysed the abundance of transcripts encoding *ptxABCD* and *phnYZ* in cDNA libraries prepared from the same Sargasso Sea samples. Significant *ptxABCD* and *phnYZ* expression was found in the 100 m sample (Table S2), with higher expression of the genes encoding the ABC transporter components (~2 cDNA reads/gDNA reads for each query), compared with that of the enzyme-encoding genes. Expression was not detected in the 50 m sample, despite the presence of *ptxABCD* and *phnYZ* in the corresponding metagenomic library. These results demonstrate that the *Prochlorococcus ptx* and *phn* genes are not only abundant but also expressed in the natural deep chlorophyll maximum populations in the Sargasso Sea.

To further our understanding of the role of the *Prochlorococcus ptxABCD, phnYZ* and *phnD* genes in other oceanic regions, we analysed their abundance in the surface water samples of the Global Ocean Survey (GOS) (Rusch *et al*., 2007). Again, the significance cut-off values (expectation 1×10^{-50}, 85% identity) were chosen to limit our analyses to *Prochlorococcus* sequences. While *Prochlorococcus recA* and *phnD* genes were present in most open ocean samples, the *ptxABCD* and *phnYZ* genes were abundant in the Sargasso Sea and the Caribbean Sea, but undetectable in the Eastern Tropical Pacific Ocean and Indian Ocean where P availability is

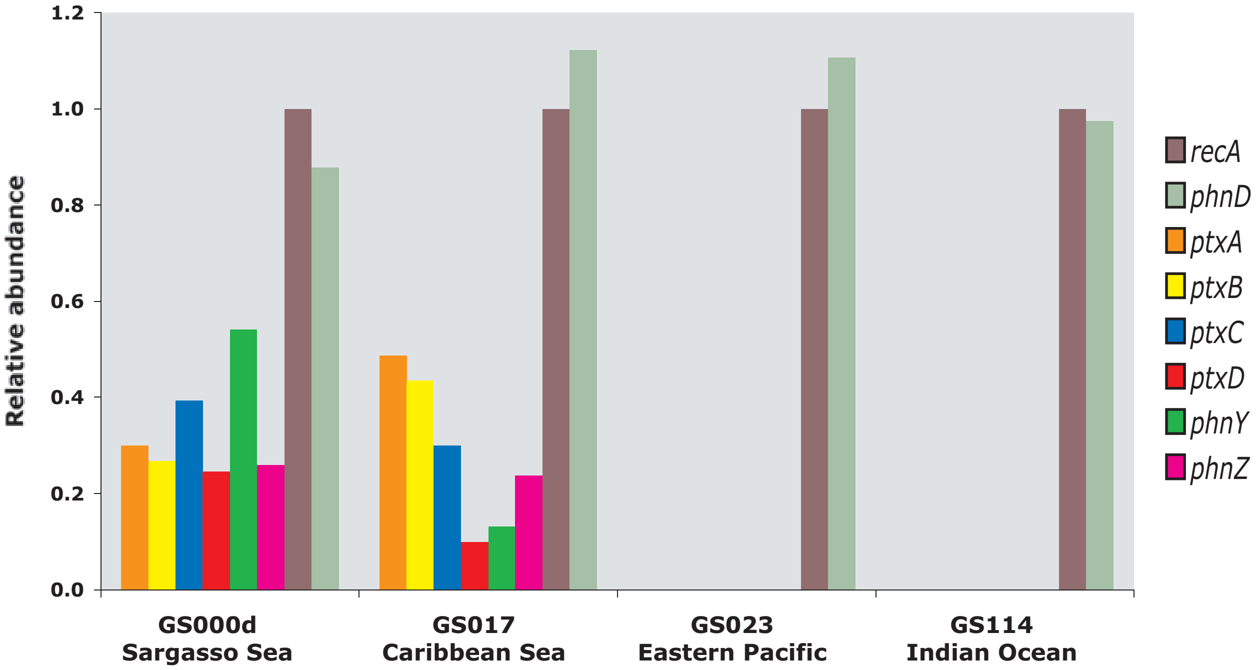

Fig. 7. *Prochlorococcus* MIT9301 *ptxABCD, phnYZ, phnD* and *recA* gene abundance in representative samples from four major ocean regions in the GOS database. Other samples from these regions showed the same trends. The number of hits to each query (expectation value e < 1e-50, identity > 80%) was size normalized using the amino acid length of the query protein. Relative abundance was estimated assuming that *recA* is present in single copy in all *Prochlorococcus* strains. Estimated regional phosphate concentrations as reported in Martiny and colleagues (2009) were 0.06 µM (GS000d, Sargasso Sea), 0.11 µM (GS017, Caribbean Sea), 0.95 µM (GS023, Eastern Tropical Pacific) and 0.17 µM (GS114, Indian Ocean).

relatively higher (Martiny *et al*., 2009) (Fig. 7). The estimated abundance of the *ptxABCD* and *phnYZ* genes relative to the single copy core gene recA was 0.25–0.54 in Sargasso Sample GS000d, and 0.10–0.49 in the Caribbean Sea sample GS017 (Fig. 7), suggesting that roughly a quarter of the *Prochlorococcus* cells in these samples contain them. Other *Prochlorococcus* P-acquisition genes have been found in high abundance in the same samples (Martiny *et al*., 2009).

Discussion

The presence of phosphite in the P pool and its utilization by marine microbes has not been previously explored, despite recent evidence that phosphite is relatively stable under aerobic conditions, has been detected in a variety of environmental samples (Hanrahan *et al*., 2005; Pasek, 2008), and that some microbes in other environments can use it as their sole P source (White and Metcalf, 2007). The concentration of phosphite in the marine environment is not known because phosphite is not specifically identified using the soluble reactive P, and total dissolved P, assays that are standard in oceanography. Soluble reactive P includes all compounds, including phosphate, that react with the colorimetric reagent under standard conditions (Murphy and Riley, 1962). This pool is dominated by phosphate but includes other organic acid-labile compounds that hydrolyse under the acidic conditions of the assay (Thomson-Bulldis and Kar, 1998). Total dissolved P is determined by a second soluble reactive P assay after complete hydrolysis by chemical means (persulfate digestion) or UV photolysis, and dissolved organic P (DOP) is obtained by subtracting soluble reactive P from the total dissolved P. Morton and colleagues (Morton *et al*., 2003) have shown that phosphite was not quantified as soluble reactive P, but that all the P of phosphite was detected after persulfate treatment. Therefore, if phosphite were present in ocean waters, it would be incorrectly classified as organic P in standard analyses. Thus the concentration of phosphite in the oceans is unknown and awaits the application of methods specifically designed to measure its concentration. Nevertheless, we have shown that *Prochlorococcus* MIT9301 can grow with phosphite as the sole P source, which to our knowledge, is the first report of phosphite utilization by a marine microbe. The presence of highly conserved *ptxABCD* phosphite utilization clusters in the genomes of other marine bacteria implies that this capability is probably not unique to MIT9301, and that phosphite might indeed play a previously unrecognized role in the ocean environment.

In *Prochlorococcus*, accessory P-acquisition genes cluster within hypervariable genomic regions, and their abundance is not congruent with rRNA phylogeny but rather with environmental P availability (Martiny *et al.*, 2006; Kettler *et al.*, 2007; Coleman and Chisholm, 2010). This is also the case for the phosphite utilization cluster: the *ptxABCD* and *phnYZ* genes are present in only two of the 18 *Prochlorococcus* genomes currently available, and the cluster is found in a hypervariable genomic island in MIT9301, suggestive of lateral gene transfer (Coleman and Chisholm, 2010; Martinez *et al.*, 2010). Our results also show that the abundance of phosphite utilization genes in natural populations is high in areas of low phosphate: approximately one quarter of *Prochlorococcus* appear to harbour these genes in the low-P Sargasso and Caribbean Seas. Thus, phosphite utilization appears to follow the same trend as many other P utilization genes, such as those encoding high-affinity transporters, alkaline phosphatase, and polyphosphate and phosphonate use, among others, which have been found at higher frequency for *Prochlorococcus* and/or for other bacterial strains in the more P-limited surface waters (Martiny *et al.*, 2006; 2009; Coleman and Chisholm, 2010; Feingersch *et al.*, 2010; Martinez *et al.*, 2010; Temperton *et al.*, 2011). Interestingly, in the depth profile series analysed here which was collected while the Sargasso Sea waters were stratified, the *ptx* gene abundance was highest in the 100 m sample, and *ptx* gene expression was detectable only at this depth. This finding suggests the possibility that the reduction carried out by the PtxD NAD-dependent phosphite dehydrogenase could furnish not only phosphate, but also reducing power that could provide energy which may be advantageous to the cells under these lower light conditions.

Despite the presence of two putative phosphonate utilization clusters (the sporadic *phnYZ* genes present in MIT9301 and MIT9303, and the unlinked *phnCDE* gene cluster located in all the sequenced strains of *Prochlorococcus*), we and others have been unable to demonstrate that *Prochlorococcus* strains can use either 2-AEPn or other phosphonates as a P source (Moore *et al.*, 2005; Martiny *et al.*, 2006). Expression of the *phnD* gene does not appear to be responsive to P limitation in *Prochlorococcus* MED4 cultures or in natural *Prochlorococcus* populations *in situ* (Martiny *et al.*, 2006; Ilikchyan *et al.*, 2010), consistent with our finding that the abundance of this gene showed no environmental clustering with P abundance. These results, together with the lack of linkage to genes encoding known phosphonate utilization enzymes, leaves open the possibility that the putative *phnCDE* phosphonate transporter genes may actually be used for other purposes. The presence of the *phnYZ* cluster in MIT9301 did not permit growth of MIT9301 using 2-AEPn or two related phosphonates, despite the fact that the abundance of this cluster is correlated with low phosphate availability and its transcription was responsive to phosphate limitation in cultures (Coleman and Chisholm, 2010; Martinez *et al.*, 2010). Further, the MIT9301 *phnYZ* genes did not enable phosphonate utilization when expressed in *E. coli*. A possible explanation for these results is that the MIT9301 enzymes may have a different substrate specificity, which is possible considering the degree of similarity of these genes to previously characterized *phnYZ* genes (37% and 33% identity at the amino acid level for *phnY* and *phnZ* respectively) (Martinez *et al.*, 2010). This explanation is plausible, since details of the biochemical reactions carried out by PhnY and PhnZ remain uncharacterized and the chemical identity of the abundant marine phosphonate pool is largely unknown. Although their function in MIT9301 remains to be defined, the high abundance and expression of *phnYZ* genes in the natural *Prochlorococcus* population of the Sargasso Sea suggests that these genes are likely to play a significant role under low-P conditions.

In summary, our results strongly suggest that phosphite utilization capability may confer a selective advantage to microbes living in low phosphate waters. The presence and expression of these genes in strains of *Prochlorococcus*, the most numerous primary producer in oligotrophic waters, provides evidence for a P-redox cycle in the marine environment and could have a profound impact on the ocean's P cycle.

Experimental procedures

Chemicals, media and bacterial strains

Sodium phosphate monobasic monohydrate (phosphate), sodium phosphite dibasic pentahydrate (phosphite), 2-aminoethylphosphonic acid, methylphosphonatic acid, phosphonoformic acid, phosphonoacetatic acid, phosphonoalanine and sodium hypophosphite monohydrate were from Sigma Aldrich (St. Louis, MO). 2-Phosphonobutyric acid, 2-phosphonopropionic acid and 3-phosphonopropionic acid were from Alfa Aesar (Ward Hill, MA). MOPS minimal medium was purchased from Tecknova (Hollister, CA). Phosphate Colorimetric Assay kit was from BioVision (Mountain View, CA). *Escherichia coli* BW16787 and BW18812 were obtained from B. Wanner through the *E. Coli* Genetic Stock Center.

Growth of Prochlorococcus *on different P sources*

All *Prochlorococcus* cultures were axenic (free of heterotrophic bacteria), and were grown at 22°C under constant light provided by cool-white fluorescent lamps at irradiances of ~25 mol Q $m^{-2} s^{-1}$ for MED4, and ~16 mol Q $m^{-2} s^{-1}$ for MIT9301 and MIT9313, in Pro99 medium, consisting of sterile (0.2 μm filtered, autoclaved) Sargasso Sea water supplemented with 800 μM NH_4Cl, trace metals, and, where appropriate, 50 μM P source (Moore *et al*., 2007). Growth was monitored using Turner Design fluorometer 10-AU to measure bulk chlorophyll fluorescence, used as a proxy for culture cell density at least during log phase growth (Moore *et al*., 2007).

Cultures to test growth in different P sources were started from mid-logarithmic cultures in phosphate-containing Pro99 medium. To replace or remove a P source, *Prochlorococcus* cultures were collected on polycarbonate filters (0.2 μm pore size), filters were washed extensively with Pro99 medium with no P source, and cells were recovered from filters and resuspended in new medium containing no P, phosphate ($NaH_2PO_4{\cdot}H_2O$), phosphite ($Na_2HPO_3{\cdot}5H_2O$) or 2-aminoethylphosphonate (50 μM) as indicated. All growth conditions were tested in duplicate cultures. Phosphate levels in the phosphite-containing medium were monitored throughout the course of the experiments using a Phosphate Colorimetric Assay (Biovision) which has a detection limit of 0.5 μM under our conditions.

Cloning of Prochlorococcus *MIT9301* ptxD *and* phnYZ

The MIT9301 *ptxD* gene was PCR-amplified using forward and reverse primers 5′-CGGCATATGAAGAAGGTTGTTATTTCCAATAAAGTT-3′, and 5′-CCGGGGATCCTTAATGATATAAAAAGTTGATAATATTTTGAGCAG-3′ respectively. The MIT9301 *phnY* and *Z* genes were amplified as a single DNA fragment using forward primer 5′-CGGCATATGAACAATATAAAATTAAAATTCGATAATGACG-3′ and reverse primer 5′-CCGGGGATCCTTAATAGATAGCTAATCTTTCGGCAACAAT-3′. Both primer sets were designed to include an upstream NdeI site and a downstream BamHI site. Using Invitrogen's TOPO-TA cloning kit, amplified fragments were then ligated downstream of the *lac* promoter in vector pCR4-TOPO (Invitrogen) and transformed into *E. coli*. The sequence of the cloned genes and their orientation with respect to the *lacZ* promoter was verified by DNA sequencing. For each construct, a recombinant plasmid with the P utilization gene in the correct orientation for P*lac*-dependent expression (forward), and a plasmid with the gene in the opposite orientation (reverse) as a negative control were chosen for complementation analysis.

Complementation analysis of Prochlorococcus *MIT9301* ptxD*,* phnY *and* phnZ *genes in* E. coli

To evaluate the ability of the cloned genes to allow growth on different P sources, recombinant plasmids were transformed into *E. coli* BW16787 (*phoA*, Δ*phnH–P*) (Lee *et al*., 1992), and transformants were streaked in MOPS medium containing 0.4% glycerol, 25 μg ml^{-1} kanamycin and 0.2 mM P source (phosphate, phosphite or 2-aminoethylphosphonate). Growth was evaluated visually after a 3-day incubation at 30°C.

P specificity tests in E. coli

Phosphorus specificity was evaluated in microtitre plate liquid cultures with different P sources as previously described (Martinez *et al*., 2010). Briefly, growth of *E. coli* BW16787 strains containing plasmids with recombinant *phnYZ* or *ptxD* plasmids was tested in MOPS medium containing 0.4% glycerol, 25 μg ml^{-1} kanamycin and 0.2 mM P source as indicated. Growth on each P source was determined by the optical density (600 nm) of the triplicate cultures after 4 days of incubation at 30°C.

Phylogenetic analysis of ptxB *and* ptxD *genes*

Homologues of the *Prochlorococcus* MIT9301 PtxD and PtxB (Accession No. YP_00109477 and YP_001091475 respectively) were identified by comparing each gene against the non-redundant NCBI database using BLASTP (Altschul *et al*., 1997). Peptides matching the query sequence with expectation values below 1×10^{-50} were considered significant and were used in subsequent analyses. For PtxB, representatives of other more distantly related predicted periplasmic phosphonate-binding proteins were considered in the analysis. These included representatives of the predicted PhnD proteins found in all *Prochlorococcus* and *Synechococcus* strains (*Prochlorococcus* MIT9301, YP_001090950; *Prochlorococcus* MED4, NP_892791; *Prochlorococcus* NATL1A, YP_001014553; and *Synechococcus*. sp. CC9605, YP_381603), as well as selected PhnD proteins from C–P lyase encoding clusters (*T. erythraeum* IMS101, YP_724380; *Cyanothece* sp. CCY0110, ZP_01730764; *P. stutzeri* WM88, AAR91732; and *E. coli str.* K-12, AP_004606). Peptide sequences were aligned using CLUSTALW2 (Chenna *et al*., 2003). The hypervariable N-terminal signal peptide was masked from the PtxD alignment (aa 1–29, MIT9301 PtxD numbering) for phylogenetic analyses. Maximum likelihood phylogenetic trees were constructed using PhyML (Guindon and Gascuel, 2003) with 100 bootstrap resamplings to determine branch support.

Sample collection, cDNA preparation and pyrosequencing

Bacterioplankton samples were collected at the Hawaii Ocean Time-series Station ALOHA in the North Pacific (22°44′N, 158°2′W) and BATS Station in the Sargasso Sea (31°40′N, 64°10′W). At each site, samples for genomic DNA and RNA extractions were collected from the photic zone in the mix layer, just below the mixed layer, and at the deep chlorophyll maximum (25, 75 and 110 m for HOT186, and 20, 50 and 100 m for BATS216), and at the mesopelagic zone (500 m). Community genomic DNA samples have been previously described (Coleman and Chisholm, 2010; Martinez *et al*., 2010). RNA extractions, bacterial ribosomal RNA subtraction, cDNA synthesis and pyrosequencing were performed according to Stewart and colleagues (2010). 454 metagenomic and metatranscriptomic data have been deposited in the Short Reads Archive (NCBI) under the following accession numbers: SRX007372 (HOT186_25m_DNA), SRX007369 (HOT186_75m_DNA), SRX007370 (HOT186_110m_DNA), SRX007371 (HOT186_500m_DNA), SRX008032 (BATS216_20m_DNA), SRX008033 (BATS216_50m_DNA), SRX008035 (BATS216_100m_DNA), SRX007384 (BATS216_500m_DNA), SRX016882

(BATS216_20m_cDNA), SRX016883 (BATS216_50m_cDNA) and SRX016884 (BATS216_100m_cDNA).

Bioinformatics

Abundance and distribution of *Prochlorococcus* MIT9301 phosphite and phosphonate utilization genes in metagenomic pyrosequencing libraries was determined as follows. Deduced peptide sequences of PtxA, PtxB, PtxC, PhnY, PhnZ, PhnD and RecA were used as a query to interrogate the metagenomic databases using NCBI BLASTX. To restrict the results of the search to closely related sequences, stringent cut off values of a bit score of 50 (Stewart *et al*., 2010; 2011) and 85% identity were applied. Abundance of a query gene (the number of reads matching the query expressed as a percentage of the total number of reads, with exact duplicates and rRNA reads removed) and expression ratio (abundance RNA/abundance DNA) were calculated as in Stewart and colleagues (2011). The abundance of *Prochlorococcus* phosphite and phosphonate utilization genes in the GOS database was analysed as above, except that an expectation value of 1×10^{-50}, and 85% identity were used as significance cut-off values. To estimate the fraction of microbes containing a gene, gene counts were size normalized using the query protein length and the percentage of microbes containing each gene was calculated assuming that *recA* is present as a single-copy gene in all bacteria (Howard *et al*., 2008; Reisch *et al*., 2008; Martinez *et al*., 2010).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Amino acid sequence alignment of *Prochlorococcus* MIT9301 PtxD with previously characterized NAD-dependent phosphite dehydrogenases. Conserved amino acid residues involved in substrate binding or catalysis in *P. stutzeri* WM88 phosphite dehydrogenase (Fodor, 1997; Woodyer *et al.*, 2003; 2005; Relyea and van der Donk, 2005) are marked in yellow. The G-X-G-X_2-GX_{17}-D motif characteristic of the Rossman fold of HA hydroxyacid dehydrogenases (Rossmann *et al.*, 1974; Wierenga *et al.*, 1985; Woodyer *et al.*, 2003) is marked in purple.

Fig. S2. Growth of *Prochlorococcus* strain MIT9301 with different concentrations of P source. *Prochlorococcus* strain MIT9301 (axenic) was grown to mid-logarithmic phase in phosphate-containing Pro99 medium, washed on filters, then resuspended in Pro99 medium containing phosphite (left panel) or phosphate (right panel) as the sole P source at the indicated concentrations. Error bars represent the standard deviation of the mean relative fluorescence of duplicate cultures.

Fig. S3. Vertical nutrient distribution for the depth profiles of the North Pacific Hawaii Ocean Time Series HOT and Sargasso Sea Bermuda Atlantic Series BATS stations at the time the of sampling (October 2006). Concentration of phosphate (blue), and nitrate plus nitrite (red) are expressed as μmol kg^{-1}. Data were obtained from Hawaii Ocean Time Series (http://hahana.soest.hawaii.edu/hot/hot-dogs/interface.html) and the Sargasso Sea Bermuda Atlantic Series websites (http://bats.bios.edu/index.html) respectively.

Table S1. Best BLASTP hits for *Prochlorococcus* MIT9301 phosphonate/phosphite-related proteins in NCBI non-redundant protein database.

Table S2. Abundance and expression of *Prochlorococcus* phosphite and phosphonate genes in Sargasso Sea and North Atlantic pyrosequencing libraries.

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