

ORIGINAL ARTICLE

The alkaline phosphatase PhoX is more widely distributed in marine bacteria than the classical PhoA

Marta Sebastian and James W Ammerman¹

Institute of Marine and Coastal Sciences, Rutgers University, New Brunswick, NJ, USA

Phosphorus (P) is a vital nutrient for all living organisms and may control the growth of bacteria in the ocean. Bacteria induce alkaline phosphatases when inorganic phosphate (P_i) is insufficient to meet their P-requirements, and therefore bulk alkaline phosphatase activity measurements have been used to assess the P-status of microbial assemblages. In this study, the molecular basis of marine bacterial phosphatases and their potential role in the environment were investigated. We found that only a limited number of homologs to the classical *Escherichia coli* alkaline phosphatase (PhoA) were present in marine isolates in the *Bacteroidetes* and γ -*proteobacteria* lineages. In contrast, PhoX, a recently described phosphatase, was widely distributed among diverse bacterial taxa, including *Cyanobacteria*, and frequently found in the marine metagenomic Global Ocean Survey database. These taxa included ecologically important groups such as *Roseobacter* and *Trichodesmium*. PhoX was induced solely upon P-starvation and accounted for approximately 90% of the phosphatase activity in the model marine bacterium *Silicibacter pomeroyi*. Analysis of the available transcriptomic datasets and their corresponding metagenomes indicated that PhoX is more abundant than PhoA in oligotrophic marine environments such as the North Pacific Subtropical Gyre. Those analyses also revealed that PhoA may be important when *Bacteroidetes* are abundant, such as in algal bloom episodes. However, PhoX appears to be much more widespread. Its identification as a gene that mediates organic P acquisition in ecologically important groups, and as a marker of P_i-stress, constitutes an important step toward a better understanding of the marine P cycle.

The ISME Journal (2009) 3, 563–572; doi:10.1038/ismej.2009.10; published online 12 February 2009

Subject Category: microbial ecology and functional diversity of natural habitats

Keywords: microbials; marine bacteria; marine phosphorus cycle; molecular markers; phosphatases; phosphorus stress

Introduction

Microbes drive the fluxes of energy and matter in the ocean. In the upper layer of the oceans dissolved inorganic phosphorus (P_i) is usually depleted, and growth and production of planktonic microbes can be stressed by P_i availability (Clark *et al.*, 1998; Wu *et al.*, 2000; Mills *et al.*, 2004; Thingstad *et al.*, 2005). Thus, to meet their phosphorus (P) requirements, bacterioplankton have developed different strategies to adapt to low or variable P_i concentrations in the environment. Strict oligotrophs, such as *Prochlorococcus* and *Pelagibacter*, have adapted to efficiently exploit very low nutrient conditions by

reducing their P-requirements (Dufresne *et al.*, 2005; Giovannoni *et al.*, 2005; Van Mooy *et al.*, 2006), which allows them to reach high numerical abundance in the open ocean. In contrast, the so-called ‘opportunistic-trophs’ (Polz *et al.*, 2006) have more versatile lifestyles and can adapt to variable P_i concentrations, exploiting localized nutrient patches or initiating a starvation response when P_i becomes insufficient.

Despite growing evidence of P_i-depletion in the ocean, studies dealing with P-starvation in marine bacterial isolates are scarce (Nystrom *et al.*, 1990; Scanlan and Wilson 1999), and it is assumed that they have the same mechanisms as *Escherichia coli*, in which the P-starvation response has been extensively studied. Under P_i-depletion, *E. coli* induces various components of the Pho regulon, a suite of genes that code for proteins required for scavenging P_i or for the use of alternative P sources (such as phosphate esters and phosphonates). A major member of this regulon in *E. coli* is PhoA, a well-characterized alkaline phosphatase that hydrolyzes

Correspondence: M Sebastian, Institute of Marine and Coastal Sciences, Rutgers University, 71 Dudley Rd, New Brunswick, NJ 08901-8521, USA.

E-mail: sebastian@marine.rutgers.edu

¹Current address: School of Marine and Atmospheric Sciences, Stony Brook University, Stony Brook, NY 11794, USA.

Received 15 December 2008; revised 13 January 2009; accepted 13 January 2009; published online 12 February 2009

phosphate esters and is induced several hundred fold when P_i becomes deficient (Wanner, 1996).

Marine bacteria also induce alkaline phosphatases when P_i is in short supply and therefore, bulk alkaline phosphatase activity measurements have been used to estimate P_i -deficiency in microbial communities (Cotner *et al.*, 1997; Van Wambeke *et al.*, 2002). However, we know little about the molecular basis of marine bacterial phosphatases, and the genes responsible for the activities observed are still unknown. A recent *in silico* study (Su *et al.*, 2007) shows that several cyanobacterial species contain putative PhoA genes, but these genes appear to belong to different families and have little homology to the *E. coli* *phoA*, raising the question of whether these genes encode P_i -regulated alkaline phosphatases.

Bacterial assemblages are composed of individual species populations with a wide range of metabolic activities, which most likely experience different levels of P-stress, depending on their P-requirements. Hence, knowledge of the genes that encode for phosphatases is crucial to develop molecular markers to address the P-status of individual groups. Intriguingly, although homologs to the *E. coli* Pho regulon genes, such as the high-affinity inorganic phosphorus transporter (PstS) and genes involved in phosphonate metabolism, have been found in marine metagenomic studies (Rusch *et al.*, 2007), homologs of the *E. coli* PhoA have not been reported, suggesting other phosphatases might prevail in marine systems.

Recently, a novel phosphatase gene (*phoX*) was identified in *Vibrio cholerae* (Majumdar *et al.*, 2005). Although still poorly characterized, PhoX is an alkaline phosphatase and a member of the Pho regulon (von Kruger *et al.*, 2006), but shares no homology to PhoA. In contrast to PhoA, which is a homodimer activated by Zn^{2+} and Mg^{2+} , PhoX is a monomeric enzyme activated by Ca^{2+} (Roy *et al.*, 1982; Majumdar *et al.*, 2005). Furthermore, PhoA is secreted to the periplasm or the extracellular space through the Sec pathway (Kim and Wyckoff, 1991), whereas PhoX is exported through the twin arginine transport system, which is able to translocate fully folded proteins across membranes (Monds *et al.*, 2006). Within the last 2 years *phoX* has been also shown to encode for the major inducible phosphatase in the soil isolate *Pseudomonas fluorescens* (Monds *et al.*, 2006), and in the pathogens *Pasteurella multocida* X-73 (Wu *et al.*, 2007) and *Campylobacter jejuni* (van Mourik *et al.*, 2008).

To develop molecular markers to assess the P-status of individual populations of bacteria, we investigated the role of PhoA and PhoX in the environment. We first analyzed the distribution of both phosphatases in marine bacteria isolates and in the marine metagenomic Global Ocean Survey (GOS) database (Rusch *et al.*, 2007). Having identified PhoX as more widespread and potentially important in marine systems, we investigated

the regulation of PhoX in marine isolates and its contribution to the phosphatase activity. Finally, we examined the expression of both phosphatases in the two marine metatranscriptomic datasets available to date, from the North Pacific Subtropical Gyre (NPSG) (Frias-Lopez *et al.*, 2008) and a coastal marine mesocosm experiment (Gilbert *et al.*, 2008).

Materials and methods

Isolation of environmental DNA

Sargasso Sea water was collected from 40 m from the Bermuda Atlantic Time Series station (31°50'N, 64°10'W) on 19 May 2006. Chesapeake Bay water was collected from the surface on 27 April 2004. Water samples for DNA analysis (500 ml–10 l) were filtered through 0.2 µm Sterivex-GP filter capsules (Millipore Corporation, Billerica, MA, USA). Samples were stored at –80 °C until extraction. DNA was extracted using the Genomic DNA Purification Kit (Puregene, Qiagen Inc., Valencia, CA, USA).

phoX clonal library construction

Degenerate PCR primers for *phoX* (Supplementary Table 1) were designed against the most conserved region based on the alignment of available nucleotide sequences. Primers were tested with the following strains: *V. cholerae*, *V. fischeri*, *V. alginolyticus*, *Vibrio* sp. TW-6, *Shewanella* sp., *Psychrobacter* sp. 760D, *Moritella* sp. 762G, *Silicibacter pomeroyi* DSS-3, *Roseovarius nubinhibens* ISM and *Roseobacter denitrificans*. PCR was performed using 10 ng of DNA in a final reaction mixture (25 µl) containing 0.2 mM dNTP, 0.4 µM of each 5' and 3' primer, 1.4 mM $MgCl_2$, 1 × PCR buffer and 1.25 µl of Taq DNA polymerase (Bio-Rad Laboratories, Hercules, CA, USA). PCR reactions were carried as follows: one cycle of 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min and one cycle of 72 °C for 10 min. PCR products were gel purified using QIAquick spin columns (Qiagen Inc., Chatsworth, CA, USA). Amplified *phoX* fragments were cloned into pCR4-TOPO using a TA cloning kit (Invitrogen, Carlsbad, CA, USA). Fifty to 100 clones were screened by PCR, using the corresponding *phoX* forward primer and the T3 primer (Supplementary Table 2). PCR products were cut with *AluI*. In the few cases where *AluI* did not cut a second digestion with *HaeIII* was performed. Clones from each library showing unique restriction fragment length polymorphism patterns were selected and sequenced using the ABI 3100-Avant sequencer (Applied Biosystems, Chicago, IL, USA).

Identification of *phoA* and *phoX* homologs and phylogenetic analysis

phoA from *E. coli* and *phoX* from *V. cholerae* were used in BLASTX to identify homologs in genome

sequences (NCBI-nr) and marine metagenomic libraries (GOS database; Rusch *et al.*, 2007). An *e*-value $<10^{-35}$ was used as the cutoff value. An amino-acid alignment of these sequences along with those obtained by PCR in this study was generated using ClustalW. PhoX sequences were trimmed to ~200 amino acids that represented the length of *phoX* fragments amplified by PCR from the environment. PhoA sequences were trimmed to ~400 amino acids. Only the metagenomic sequences that covered the complete fragment were included in the alignments. The resulting alignment was then used to realign (back translate) nucleotide sequences and this nucleotide alignment was used to generate a maximum likelihood tree with the PhyML program (Guindon and Gascuel, 2003) using the nucleotide substitution model GTR (General time reversible model). All the positions in the alignment were included in the analysis. MEGA4 was used to draw and edit the tree.

Homologs in the marine transcriptomic databases (Frias-Lopez *et al.*, 2008; Gilbert *et al.*, 2008) were identified by TBLASTX using the PhoA and PhoX sequences obtained from NCBI-nr and GOS database as a query. The taxonomic affiliation of sequences was later analyzed by alignment against the NCBI-nr database using the BLASTX algorithm.

P-starvation experiments: time course of alkaline phosphatase activity and expression of *phoX* in marine isolates

S. pomeroyi and *R. denitrificans* were grown in 11 Marine Broth diluted 1:9 with filtered seawater supplemented with 10 mM glucose, 9 mM NH₄Cl, 200 μM of K₂HPO₄, 1 μM Fe and 1 ml/l vitamin solution (2 mg biotin, 2 mg folic acid, 10 mg pyridoxine HCl, 5 mg riboflavin, 5 mg thiamine, 5 mg nicotinic acid, 0.1 mg cyanocobalamin, 5 mg *p*-aminobenzoic acid in 100 ml of distilled water, pH 7). Cells were harvested by centrifugation at the onset of stationary phase. Half of the cells were resuspended in P-replete medium (+P, 200 μM K₂HPO₄) and the other half in medium without added P_i (-P). Samples were taken at different time points for optical density (OD), RNA and alkaline phosphatase activity assays. Experiments were independently repeated at least twice and performed in duplicate. Samples for RNA (40 ml) were centrifuged for 10 min at 12 000 × *g* and the pellets were immediately frozen in liquid nitrogen and stored at -80 °C. RNA was extracted using TRI reagent (Sigma-Aldrich, St Louis, MO, USA) and treated with Turbo DNase (Ambion Inc., Austin, TX, USA). Two μg to 3 μg of total RNA was reverse transcribed using random hexamers and the High Capacity cDNA archive kit (Applied Biosystems) according to the manufacturer's instructions. PCR was performed using primers designed to amplify internal fragments of *phoX* and *rplU* (ribosomal protein L21) of *S. pomeroyi* and *R. denitrificans* (Supplementary

Table 2). *rplU* served as a control for cDNA synthesis. Two μl of cDNA was used as template in the following reaction: 1 cycle of 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 53 °C (*phoX*) or 57 °C (*rplU*) for 30 s, 72 °C for 1 min and 1 cycle of 72 °C for 10 min. PCR products were sequenced to confirm gene of interest. Alkaline phosphatase activity was determined in triplicate subsamples by monitoring the rate of hydrolysis of the fluorogenic substrate 6, 8-difluoro-4-methylumbelliferyl phosphate (DifMUP, Invitrogen, Eugene, OR, USA) at a final concentration of 10 μM. The DifMUP produced during the assay was detected as an increase in fluorescence over time using a SpectraMax Gemini XS 96-well fluorescence microplate reader. A standard curve with DifMUP (Sigma-Aldrich) was used to quantify the hydrolysis rates (initial slope of the phosphatase assay divided by the slope of the standard curve).

phoX expression under different conditions

Additional experiments were carried out to check whether PhoX was induced under P_i-replete conditions by the presence of organic P-substrates or carbon starvation, or through a general stress response (Fe starvation). *S. pomeroyi* cells were grown in marine basal medium supplemented with 10 mM glucose (C), 200 μM of K₂HPO₄ (P_i) and 1 μM Fe. Cells were harvested by centrifugation at the exponential phase, resuspended in marine basal medium and distributed in triplicate tubes. Nutrients were added to yield the following five treatments: all three nutrients (C, P_i, Fe, '+ P_i treatment'); all three nutrients and glucose-6-phosphate as an organic P substrate ('+ DOP treatment'); C, Fe but no P_i added ('- P_i treatment'); P_i and Fe but no C added ('- C treatment'); P_i and C but no Fe added ('- Fe treatment'). Samples for OD were taken at different time intervals and samples for RNA (20 ml) were taken 5 h after inoculation. RNA extraction and RT-PCR experiments were performed following the procedures outlined above.

Generation of *phoX* knockout construct

A knockout of *phoX* was generated in *S. pomeroyi* by homologous recombination. To generate the construct, primers were designed based on the genome to amplify *phoX* from *S. pomeroyi*. *SphoX*(+): 5'-CCGGAATTCATGAAAACGACCGC ACCCTG-3' and *SphoX*(-): 5'-CTAGTCTAGATCAGC CGATCACGCCGCATC-3' contained an *Eco*RI and *Xba*I restriction sites, respectively. *phoX* was amplified from genomic DNA, digested with *Eco*RI and *Xba*I, then cloned into pUC19, which contains an ampicillin-resistance gene. Positive clones were confirmed by PCR. pUC19-*phoX* was then digested with *Age*I and *Bam*HI, which allowed a 930 bp fragment of *phoX* to be exchanged with the 940 bp kanamycin-resistance cassette, resulting in the plasmid pUC19-*phoX*-kan^R.

Gene disruption by homologous recombination

Forty ml of *S. pomeroyi* were grown overnight in half-strength Marine Broth at 30 °C to an OD₆₀₀ of approximately 0.5, chilled on ice for 20 min and centrifuged at 4000 × *g* for 15 min at 4 °C. The supernatant was removed and the pellet was concentrated 20-fold. Cells were made electrocompetent by washing three times with ice-cold 10% glycerol. Following the final wash, cells were resuspended in 10% glycerol on ice. A 40 μl sample of cells was mixed with 100 ng of the knockout construct (pUC19-*phoX-kan*^R), incubated for 1 min on ice, then electroporated at 12 kV/cm. Cells were resuspended in 2 ml of prewarmed half-strength Marine Broth and incubated for 20 h at 30 °C with shaking. Transformants were selected on half-strength Marine agar plates containing 50 μg/ml kanamycin. After 2 days at 30 °C, transformants were transferred to media containing ampicillin to confirm that homologous recombination had occurred. Growth in the presence of kanamycin, but not ampicillin, indicated that clones no longer contained the pUC19-*phoX-kan*^R vector (which contained an *amp*^R gene), and that homologous recombination was successful. To further confirm homologous recombination, genomic DNA was isolated and used as template in one PCR using primers against the flanking neighbor genes of *phoX* (flank-F:5'-CCGGCAGAGATTCTGCTAGCA-3', flank-R:5'-AGGGCTTAACCCGGATCGTCG-3') and another PCR using the primer flank-F and a reverse primer in the *kan*^R cassette. Sequencing these regions confirmed successful knockout of *phoX*.

Sequence data

The sequences reported in this study are deposited with GenBank under accession numbers EU669006-EU669012, EU669014-EU669016, EU669018-EU669020, EU669022-EU669029, EU669031-EU669071.

Results and discussion

Abundance and distribution of PhoA and PhoX in marine bacteria

To determine if PhoA is present in marine bacteria, we searched the microbial genome database (NCBI-nr) and the marine metagenomic GOS database (Rusch *et al.*, 2007) for PhoA homologs. BLAST searches revealed that only a few marine isolates in the γ -proteobacteria and *Bacteroidetes* lineage harbored a PhoA homolog (Figure 1). In addition, only 10 PhoA-like sequences were found in the GOS database (Rusch *et al.*, 2007), all of them closely related to two marine γ -proteobacterial isolates. The low abundance of PhoA homologs in the marine metagenomic database suggests PhoA may not play an important role in oceanic waters.

To investigate the role of PhoX in marine bacteria, we searched the microbial genome database and the GOS database (Rusch *et al.*, 2007), as well as

performed a PCR-based survey on samples from the Sargasso Sea and the Chesapeake Bay. BLASTX searches retrieved >90 *phoX* homologs in marine isolates, and 417 environmental *phoX*-like sequences (*e*-value <10⁻³⁵). Phylogenetic analysis of these sequences, along with 62 sequences obtained in the PCR survey, provided evidence that *phoX* was widespread among diverse bacterial phyla, including *Cyanobacteria* (Figure 2). *phoX* sequences grouped into four main clusters. The first cluster contained mostly α -proteobacterial sequences from the *Roseobacter* clade and γ -proteobacterial sequences, along with the majority of the environmental sequences. The second cluster contained representatives from diverse bacterial lineages including additional *Roseobacter* sequences, the third cluster consisted mainly of γ -proteobacterial and *Bacteroidetes* sequences, and the fourth cluster contained only cyanobacterial sequences. Although environmental sequences were numerically more abundant in the first cluster, they were found across all four clusters (Figure 2). Several marine isolates had two phylogenetically distant *phoX* homologs in their genome, which, together with the lack of correspondence between *phoX* phylogeny and the bacterial lineages (Figure 2), suggests the widespread occurrence of lateral gene transfer events. Acquisition of genes involved in nutrient assimilation seems to occur frequently in microbes (Rocap *et al.*, 2003; Dyhrman *et al.*, 2006), and studies show nutrient assimilation genes are sometimes located on genomic islands, which are hotspots for recombination (Rocap *et al.*, 2003). Indeed *V. cholerae phoX* is located on chromosome II, which encodes many stress-related proteins and a gene-capture system involved in horizontal gene transfer (Heidelberg *et al.*, 2000).

Regulation of PhoX in marine isolates

We next examined whether *phoX* is functional in marine isolates using two members of the *Roseobacter* clade, one of the most abundant bacterial groups in diverse marine habitats (Moran *et al.*, 2007). *S. pomeroyi* DSS-3 and *R. denitrificans* both lack PhoA in their genome. *S. pomeroyi* has one *phoX* homolog on the chromosome (*phoX*^{Sc}), whereas *R. denitrificans* has two distinct homologs of *phoX*, one on the chromosome (*phoX*^{Rc}) and one on the megaplasmid pTB1 (*phoX*^{Rp}). The proteins encoded by *phoX*^{Sc} and *phoX*^{Rc} share only 38% amino-acid identity. We subjected cells of these two strains to abrupt P-starvation and studied the response of alkaline phosphatase activity and *phoX* gene expression (Figure 3). The time-course response to P-starvation was similar in both strains. Alkaline phosphatase activity was elevated 44-fold (*S. pomeroyi*) and 90-fold (*R. denitrificans*) within 5 h after inoculation into P_i-free medium, whereas activity in cells resuspended in P_i-replete medium remained almost undetectable (Figure 3a). When P_i

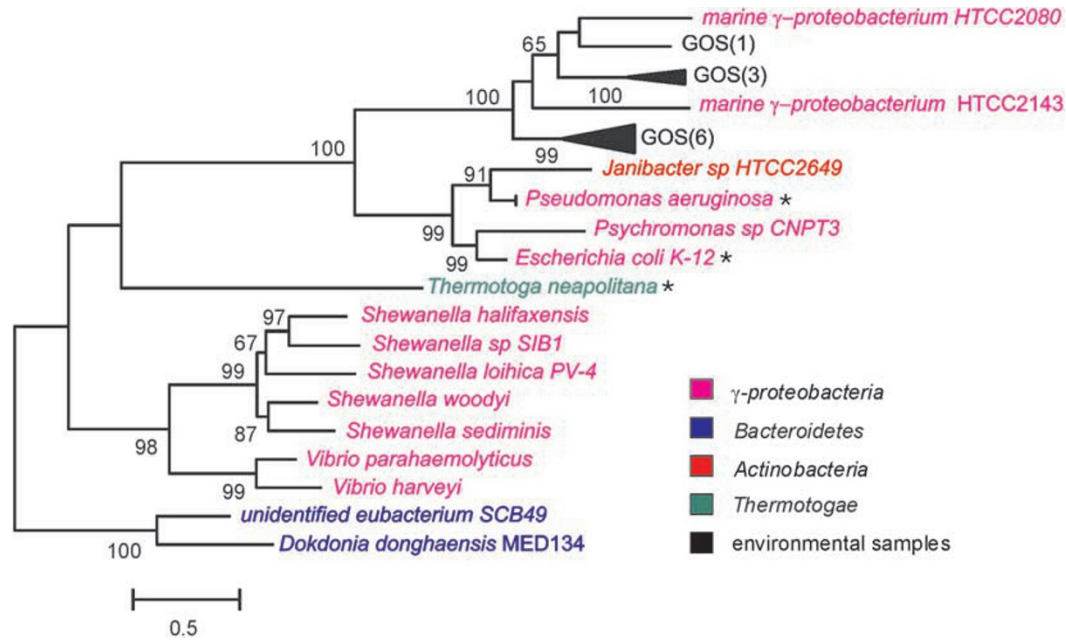


Figure 1 Phylogenetic relationships of *phoA* homologs from marine bacterial isolates and marine metagenomic libraries (GOS). Sequences were retrieved from NCBI by BLASTX using *E. coli phoA* (e -value $< 10^{-35}$). Non-marine isolates with confirmed phosphatase activity were included in the analyses and are marked with an asterisk. Color code indicates the bacterial lineage. The number of closely related sequences is indicated in parentheses. Tree was constructed by maximum likelihood based on the alignment of ~ 400 amino acids. Values at nodes represent the average ratio likelihood test. Scale bar represents number of substitution per site. Similar topology was obtained when neighbor-joining was used to calculate the evolutionary relationships. GOS, Global Ocean Survey.

was added back to the cultures, alkaline phosphatase activity dropped 50% within 0.5 h. Expression of *phoX^{Rc}* and *phoX^{Sc}* was induced upon P-starvation, and then decreased to undetectable levels upon P-replenishment (Figure 3b), confirming the trend observed in alkaline phosphatase activity. In contrast, *phoX^{Rp}* (the *R. denitrificans* plasmid *phoX*) was only weakly expressed throughout the experiment, with no correlation to P_i levels.

We also analyzed if PhoX is induced under conditions other than P_i -stress. On the basis of observations that most organic P compounds must be dephosphorylated before the carbon moiety can be taken up into the cell (Bengis-Garber and Kushner, 1982; Hernandez *et al.*, 1996), it has been hypothesized that bacteria could also produce phosphatases to obtain readily available carbon (Hoppe, 2003). Therefore, we carried out an experiment with *S. pomeroyi* to investigate whether PhoX expression was induced by either carbon starvation or the presence of an organic P source under P_i -replete conditions. In addition, we subjected *S. pomeroyi* cells to iron starvation to rule out the possibility of PhoX being induced through a general stress response (Figure 4). These experiments revealed PhoX is solely induced upon P_i -depletion, and validates its use as an indicator of P_i -stress.

The role of PhoX as a P-scavenging mechanism was further confirmed when *phoX^{Sc}* was inactivated by insertional mutation. Knockout mutants showed 10-fold diminished activity compared with wild type (Figure 5), indicating that PhoX was the major

phosphatase in *S. pomeroyi*, accounting for 90% of the phosphatase activity. These results are consistent with the studies on non-marine isolates where PhoX was also found to be the major phosphatase (Monds *et al.*, 2006; Wu *et al.*, 2007; van Mourik *et al.*, 2008).

Analysis of metatranscriptomic databases

Although metagenomic databases provide information about the genes present in the environment, only metatranscriptomics can be used to elucidate whether these genes are expressed, and under what conditions. Two marine metatranscriptomic datasets have been recently published, one from the oligotrophic waters of the NPSG (Frias-Lopez *et al.*, 2008) and another from a coastal marine mesocosm (Gilbert *et al.*, 2008). We analyzed both datasets for the presence of PhoA and PhoX.

In the NPSG metatranscriptome, we found no transcripts of either of these phosphatases. To determine whether the genes were present, but simply not expressed, we searched the corresponding metagenomic database and retrieved 19 homologs of *phoX*, but no homologs of *phoA*. This finding supports our previous observation with the GOS database that indicated *phoA* was less abundant than *phoX* in oceanic waters. The normalized abundance of *phoX* (relative to the single copy gene *recA*) was 18%, indicating that one in five bacterial cells in the NPSG metagenome harbored *phoX*. The lack of expression of *phoX* suggests the microbial

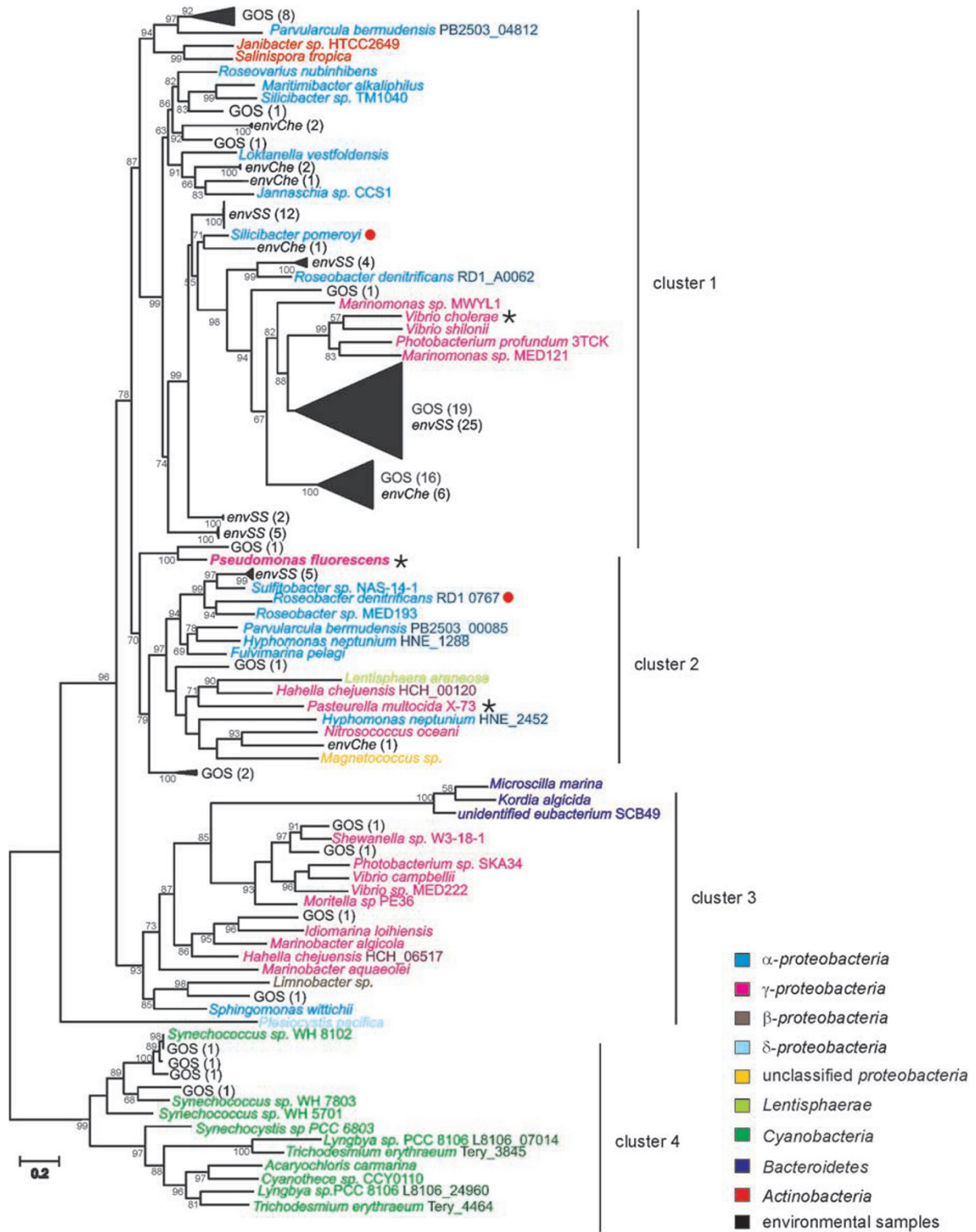


Figure 2 Phylogenetic relationships of representative *phoX* homologs from marine bacterial isolates and marine metagenomic libraries (GOS). Sequences were retrieved from NCBI by BLASTX using *V. cholerae phoX* (e -value $< 10^{-35}$). Non-marine isolates with confirmed phosphatase activity were included in the analyses and are marked with an asterisk. Color code indicates the bacterial lineage. Sequences are labeled with organism name and their ORF number when the same strain has more than one *phoX*, and the number of closely related sequences is indicated in parentheses. Sequences obtained in this study are indicated by the 'env' prefix ('envSS': Sargasso Sea, 'envChe': Chesapeake Bay). Red dots indicate genes whose expression has been shown to be activated under P_i -starvation in this study. Tree was constructed by maximum likelihood based on the alignment of ~ 200 amino acids. Values at nodes represent the average ratio likelihood test, and scale bar represents number of substitutions per site. Similar topology was obtained when neighbor-joining was used to calculate the evolutionary relationships. GOS, Global Ocean Survey.

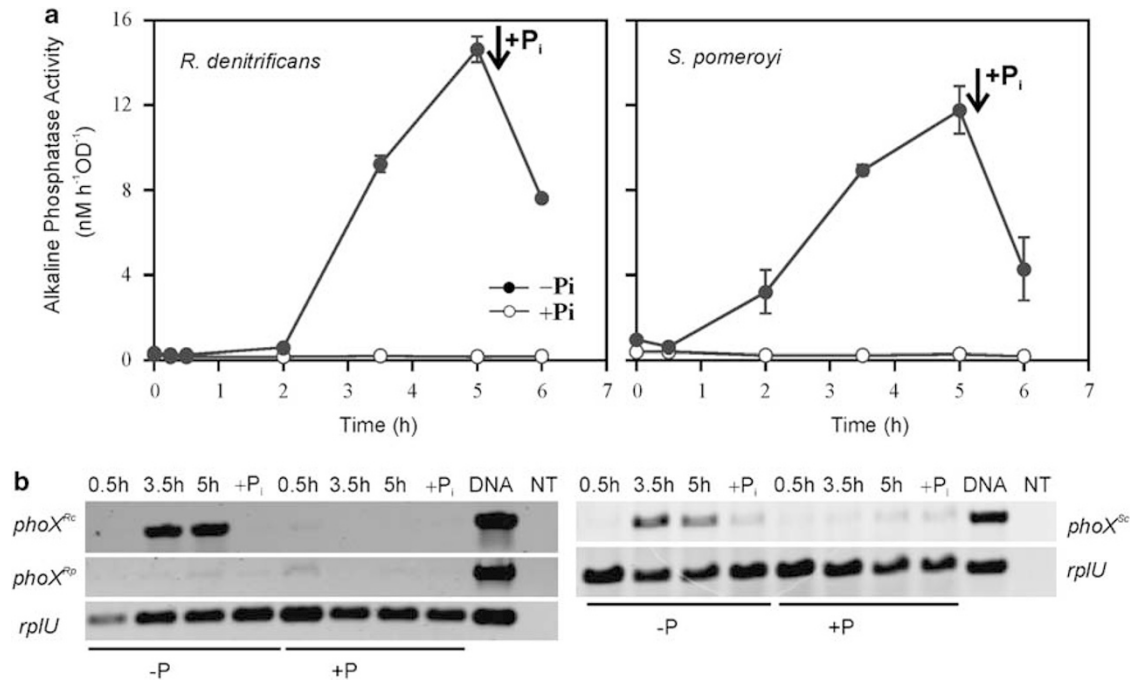


Figure 3 Time course of P-starvation response in marine isolates. (a) Induction of alkaline phosphatase activity and (b) expression of *phoX* in *R. denitrificans* and *S. pomeroyi* cells resuspended in medium with no added P (-P_i) compared with cells resuspended in P_i-replete medium (+P_i). Arrows indicate when phosphate was added back to the medium. *phoX^{sc}* (RD1_0767): *R. denitrificans* chromosome *phoX*; *phoX^{sp}* (RD1_A0062): *R. denitrificans* plasmid pTB1 *phoX*. *phoX^{sc}* (SPO1860): *S. pomeroyi* *phoX*. *rplU* transcript level was used as a Pho-independent control for cDNA synthesis. NT: no template control. No RT controls were included with every set of samples. Data points represent the average of duplicate cultures with three independent measurements for each of the cultures. Error bars represent the standard deviation. P, phosphorus; P_i, inorganic phosphorus.

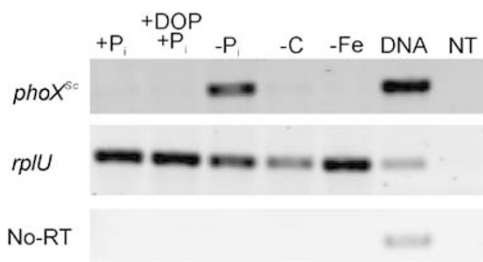


Figure 4 Expression of *phoX* in *S. pomeroyi* cells resuspended in P_i-replete medium (+P_i), P_i-replete medium supplemented with G6P as an organic P source (+DOP), P-free medium (-P_i), C-free medium (no glucose added, -C), Fe-free medium (-Fe). DNA: DNA control. NT: no template control. *rplU* transcript level was used as a Pho-independent control for cDNA synthesis. No RT controls were included with every set of samples. G6P, glucose-6-phosphate; P, phosphorus; P_i, inorganic phosphorus.

assemblage was not stressed by P_i availability, which is supported by a recent study showing that despite evidence the NPSG is moving toward P-limitation (Karl *et al.*, 2001), the microbial community is currently not P_i-stressed (Van Mooy and Devol, 2008).

The coastal marine mesocosm transcriptome was created to study the prokaryotic assemblages in a mesocosm in which a phytoplankton bloom was induced by the addition of nutrients. Samples were taken at the peak of the bloom and 5 days later when

the phytoplankton population had crashed. A total of 5 transcripts of *phoX* and 41 transcripts of *phoA* were found in the metatranscriptomic datasets, with the corresponding metagenomic datasets containing 27 and 66 homologs of *phoX* and *phoA*, respectively. These findings indicate that in this simulated eutrophic system, *phoA* was more abundant and played a more important role than *phoX*. To better understand why *phoA* was so abundant, we examined the putative taxonomic origin of these genes, keeping in mind all the following caveats, such as sequence read length, horizontal gene transfer and gene conservation across taxa. Approximately 75% of the *phoA* transcripts were similar to *phoA* found in *Bacteroidetes* (Figure 6). *Bacteroidetes* represented the fourth most abundant gene group in the mesocosm and had the third highest transcriptional activity (Gilbert *et al.*, 2008). The differences among the taxonomic distribution of *phoA* genes and transcripts suggests *Bacteroidetes* were more P_i-stressed than other bacterioplankton groups. Different components of the microbial community probably experience different degrees of P_i-stress under the same environmental conditions, depending on their cellular quota and their nutrient acquisition capacities. *Bacteroidetes* are heterotrophic bacteria frequently found on macroscopic organic particles or in nutrient-rich microenvironments associated with phytoplankton blooms (Bauer *et al.*, 2006). Thus, it is

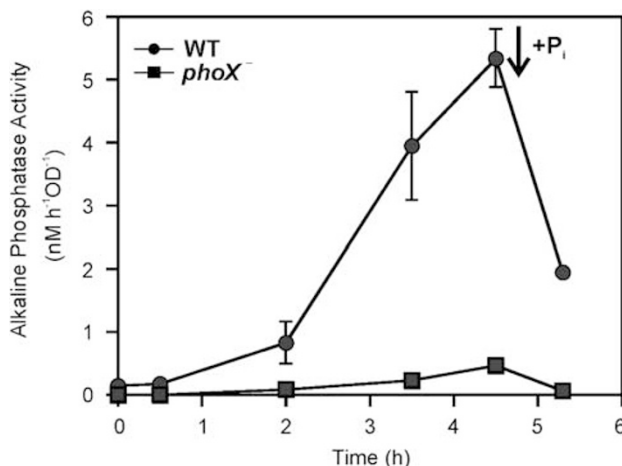


Figure 5 Time course of alkaline phosphatase activity in *Silicibacter pomeroyi* wild type (WT) and the *phoX*⁻ mutant (*phoX*⁻). The arrow indicates when inorganic phosphate (P_i) was added back to the medium. Errors bars represent the standard deviation of three replicates.

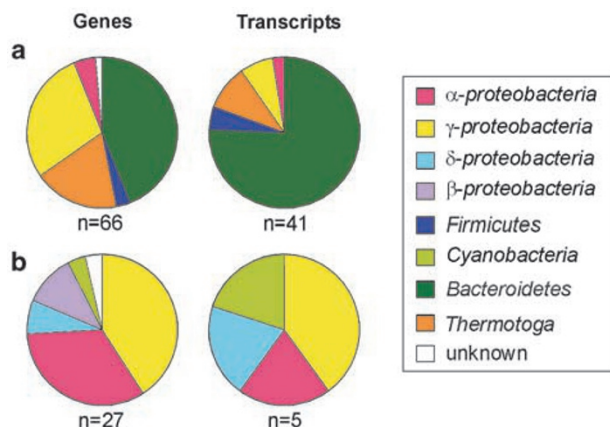


Figure 6 Heterogeneous P_i-stress response in marine bacteria. Putative taxonomic affiliation of phosphatase genes and transcripts in a coastal mesocosm experiment (Gilbert *et al.*, 2008) (a) *phoA*, (b) *phoX* (see Supplementary Tables 3 and 4 for more information).

most likely that their nutrient requirements are higher than other groups that are acclimated to less eutrophic conditions. In contrast, the small contribution of γ - and α -proteobacteria to the total number of *phoA* transcripts suggested their P-demands were lower and therefore they were less stressed by P_i availability. This might also explain the low abundance of *phoX* transcripts, as γ - and α -proteobacteria were the two most highly represented groups in the *phoX* gene homologs (Figure 6). The number of phosphatase transcripts decreased drastically after the phytoplankton bloom crashed (Table 1), most likely owing to a release of nutrients as the phytoplankton cells were degraded.

The heterogeneous P_i-stress response of different bacterial groups underlines the importance of having molecular markers to interrogate the P-status of

Table 1 Abundances for phosphatase genes and transcript homologs in the coastal marine mesocosm transcriptome (Gilbert *et al.*, 2008)

	<i>phoA</i>	<i>phoX</i>	Total no. of reads
Genes			
Peak of the bloom	24	12	343 988
Post bloom	42	15	1 302 302
Transcripts			
Peak of the bloom	38	4	293 960
Post bloom	3	1	212 393

these groups *in situ*. In this study, we have shown most marine bacterioplankton appear to use a different alkaline phosphatase (PhoX) than the classical phosphatase PhoA. PhoX is widely distributed among bacterial lineages, including *Cyanobacteria*. Its widespread distribution, together with its relatively high sequence conservation, and its regulation by P_i, make PhoX an excellent molecular marker to assess the *in situ* P-status of marine bacteria. However, we have also shown that PhoA may be essential for elucidating the P-status of certain bacterial groups, such as *Bacteroidetes*. Some marine isolates such as the unidentified *eubacterium* SCB49 or *V. parahaemolyticus* have both PhoA and PhoX encoded in their genomes. Further studies are needed to understand the advantages that each of the enzymes provides these bacteria.

The requirement of Ca²⁺ instead of Zn²⁺ for PhoX catalytic activity has probably been a major factor in its selection over PhoA in marine environments, where Zn²⁺ often occurs at subnanomolar concentrations (Lohan *et al.*, 2002). Indeed, replacement of Zn²⁺ by other elements has also been observed in other metalloenzymes as an adaptive response to oligotrophic conditions (Morel and Price, 2003). PhoX is found in ecologically important bacterial species, including common heterotrophs such as *Roseobacter*, which play an important role in the global carbon and sulfur cycle (Wagner-Dobler and Biebl, 2006), and *Cyanobacteria* such as *Trichodesmium*, a marine diazotroph that has a substantial influence in the nitrogen and carbon cycle in oligotrophic tropical and subtropical oceans (Dyhrman *et al.*, 2006). Therefore, the identification of *phoX* as a potential molecular marker of P_i-stress constitutes an important step forward toward understanding how P control of bacterial growth will affect global biogeochemical cycles.

Acknowledgements

We thank the captain and the crew of the R/V Atlantic Explorer for assistance with Sargasso sea sampling; J Sylvan for providing the Chesapeake Bay sample; MA Moran (U Georgia) and R Blakenship (Washington

University, St Louis) for providing the cultures of *S. pomeroyi* and *R. denitrificans*, respectively; J Sylvan, L McGuinness and C Vetriani for their advice on molecular techniques; K Thamatrakoln for helping in the development of the knockouts; L Kerkhof, P Falkowski and K Bidle for equipment access; and D Rusch for the help with bioinformatics. We also thank Y Helman, A Vardi, K Thamatrakoln and K Bidle for their comments on the manuscript. This study was supported in part by a postdoctoral fellowship from the Spanish Ministry of Education (to MS), a Marie Curie Outgoing European Fellowship (to MS) and a grant from NSF Biological Oceanography Program.

References

- Bauer M, Kube M, Teeling H, Richter M, Lombardot T, Allers E *et al.* (2006). Whole genome analysis of the marine *Bacteroidetes Gramella forsetii* reveals adaptations to degradation of polymeric organic matter. *Environ Microbiol* **8**: 2201–2213.
- Bengis-Garber C, Kushner DJ. (1982). Role of membrane-bound 5'-nucleotidase in nucleotide uptake by the moderate halophile *Vibrio costicola*. *J Bacteriol* **149**: 808–815.
- Clark LL, Ingall ED, Benner R. (1998). Marine phosphorus is selectively remineralized. *Nature* **393**: 426.
- Cotner JB, Ammerman JW, Peele ER, Bentzen E. (1997). Phosphorus-limited bacterioplankton growth in the Sargasso Sea. *Aquat Microb Ecol* **13**: 141–149.
- Dufresne A, Garczarek L, Partensky F. (2005). Accelerated evolution associated with genome reduction in a free-living prokaryote. *Genome Biol* **6**: R14.
- Dyhrman ST, Chappell PD, Haley ST, Moffett JW, Orchard ED, Waterbury JB *et al.* (2006). Phosphonate utilization by the globally important marine diazotroph *Trichodesmium*. *Nature* **439**: 68–71.
- Frias-Lopez J, Shi Y, Tyson GW, Coleman ML, Schuster SC, Chisholm SW *et al.* (2008). Microbial community gene expression in ocean surface waters. *Proc Natl Acad Sci USA* **105**: 3805–3810.
- Gilbert JA, Field D, Huang Y, Edwards R, Li W, Gilna P *et al.* (2008). Detection of large numbers of novel sequences in the metatranscriptomes of complex marine microbial communities. *PLoS ONE* **3**: e3042.
- Giovannoni SJ, Tripp HJ, Givan S, Podar M, Vergin KL, Baptista D *et al.* (2005). Genome streamlining in a cosmopolitan oceanic bacterium. *Science* **309**: 1242–1245.
- Guindon S, Gascuel O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* **52**: 696–704.
- Heidelberg JF, Eisen JA, Nelson WC, Clayton RA, Gwinn ML, Dodson RJ *et al.* (2000). DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* **406**: 477–483.
- Hernandez I, Hwang SJ, Heath RT. (1996). Measurement of phosphomonoesterase activity with a radiolabelled glucose-6-phosphate. Role in the phosphorus requirement of phytoplankton and bacterioplankton in a temperate mesotrophic lake. *Arch Hydrobiol* **137**: 265–280.
- Hoppe HG. (2003). Phosphatase activity in the sea. *Hydrobiologia* **493**: 187–200.
- Karl DM, Björkman KM, Dore JE, Fujieki L, Hebel DV, Houlihan T *et al.* (2001). Ecological nitrogen-to-phosphorus stoichiometry at station ALOHA. *Deep Sea Res Part II Top. Stud Oceanogr* **48**: 1529–1566.
- Kim EE, Wyckoff HW. (1991). Reaction mechanism of alkaline phosphatase based on crystal structures. Two-metal ion catalysis. *J Mol Biol* **218**: 449–464.
- Lohan MC, Statham PJ, Crawford DW. (2002). Total dissolved zinc in the upper water column of the subarctic North East Pacific. *Deep Sea Res Part II Top. Stud Oceanogr* **49**: 5793–5808.
- Majumdar A, Ghatak A, Ghosh RK. (2005). Identification of the gene for the monomeric alkaline phosphatase of *Vibrio cholerae* serogroup O1 strain. *Gene* **344**: 251–258.
- Mills MM, Ridame C, Davey M, La Roche J, Geider RJ. (2004). Iron and phosphorus co-limit nitrogen fixation in the eastern tropical North Atlantic. *Nature* **429**: 292–294.
- Monds RD, Newell PD, Schwartzman JA, O'Toole GA. (2006). Conservation of the Pho regulon in *Pseudomonas fluorescens* Pf0-1. *Appl Environ Microbiol* **72**: 1910–1924.
- Moran MA, Belas R, Schell MA, Gonzalez JM, Sun F, Sun S *et al.* (2007). Ecological genomics of marine *Roseobacters*. *Appl Environ Microbiol* **73**: 4559–4569.
- Morel FMM, Price NM. (2003). The biogeochemical cycles of trace metals in the oceans. *Science* **300**: 944–947.
- Nystrom T, Flardh K, Kjelleberg S. (1990). Responses to multiple-nutrient starvation in marine *Vibrio* sp. strain CCUG 15956. *J Bacteriol* **172**: 7085–7097.
- Polz MF, Hunt DE, Preheim SP, Weinreich DM. (2006). Patterns and mechanisms of genetic and phenotypic differentiation in marine microbes. *Phil Trans R Soc B* **361**: 2009–2021.
- Rocap G, Larimer FW, Lamerdin J, Malfatti S, Chain P, Ahlgren NA *et al.* (2003). Genome divergence in two *Prochlorococcus* ecotypes reflects oceanic niche differentiation. *Nature* **424**: 1042–1047.
- Roy NK, Ghosh RK, Das J. (1982). Monomeric alkaline phosphatase of *Vibrio cholerae*. *J Bacteriol* **150**: 1033–1039.
- Rusch DB, Halpern AL, Sutton G, Heidelberg KB, Williamson S, Yooseph S *et al.* (2007). The sorcerer II global ocean sampling expedition: Northwest Atlantic through eastern tropical Pacific. *PLoS Biol* **5**: e77.
- Scanlan DJ, Wilson WH. (1999). Application of molecular techniques to addressing the role of P as a key effector in marine ecosystems. *Hydrobiologia* **401**: 149–175.
- Su Z, Olman V, Xu Y. (2007). Computational prediction of Pho regulons in cyanobacteria. *BMC Genomics* **8**: 156.
- Thingstad TF, Krom MD, Mantoura RFC, Flaten GAF, Groom S, Herut B *et al.* (2005). Nature of phosphorus limitation in the ultraoligotrophic Eastern Mediterranean. *Science* **309**: 1068–1071.
- Van Mooy BAS, Devol AH. (2008). Assessing nutrient limitation of *Prochlorococcus* in the North Pacific subtropical gyre by using an RNA capture method. *Limnol Oceanogr* **53**: 78–88.
- Van Mooy BAS, Rocap G, Fredricks HF, Evans CT, Devol AH. (2006). Sulfolipids dramatically decrease phosphorus demand by picocyanobacteria in oligotrophic marine environments. *Proc Natl Acad Sci* **103**: 8607–8612.

- van Mourik A, Bleumink-Pluym NMC, van Dijk L, van Putten JPM, Wosten MMSM. (2008). Functional analysis of a *Campylobacter jejuni* alkaline phosphatase secreted via the Tat export machinery. *Microbiology* **154**: 584–592.
- Van Wambeke F, Christaki U, Giannokourou A, Moutin T, Souvemerzoglou K. (2002). Longitudinal and vertical trends of bacterial limitation by phosphorus and carbon in the Mediterranean Sea. *Microb Ecol* **43**: 119–133.
- von Kruger WMA, Lery LMS, Soares MR, de Neves-Manta FS, Silva C, Neves-Ferreira AGD *et al.* (2006). The phosphate-starvation response in *Vibrio cholerae* O1 and *phoB* mutant under proteomic analysis: disclosing functions involved in adaptation, survival and virulence. *Proteomics* **6**: 1495–1511.
- Wagner-Dobler I, Biebl H. (2006). Environmental biology of the marine *Roseobacter* lineage. *Annu Rev Microbiol* **60**: 255–280.
- Wanner BL. (1996). Phosphorus assimilation and control of the phosphate regulon. In: Neidhardt FC (ed). *Escherichia and Salmonella: Cellular and Molecular Biology*. ASM Press: Washington, DC, pp 1357–1381.
- Wu J, Sunda W, Boyle EA, Karl DM. (2000). Phosphate depletion in the Western North Atlantic ocean. *Science* **289**: 759–762.
- Wu J-R, Shien J-H, Shieh HK, Hu C-C, Gong S-R, Chen L-Y *et al.* (2007). Cloning of the gene and characterization of the enzymatic properties of the monomeric alkaline phosphatase (PhoX) from *Pasteurella multocida* strain X-73. *FEMS Microbiol Lett* **267**: 113–120.

Supplementary Information accompanies the paper on The ISME Journal website (<http://www.nature.com/ismej>)