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ORIGINAL ARTICLE

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

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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.
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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 'opportuni-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

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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 *Alu*I. In the few cases where *Alu*I did not cut a second digestion with *Hae*III 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 1 l Marine Broth diluted 1:9 with filtered seawater supplemented with 10 mM glucose, 9 mM NH_4Cl, 200 μM of K_2HPO_4, 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 K_2HPO_4) 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. pomeroy*i cells were grown in marine basal medium supplemented with 10 mM glucose (C), 200 μM of K_2HPO_4 (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*. S*phoX*(+): 5′-CCGGAATTCATGAAAAACGACCGC ACCCTG-3′and S*phoX*(−): 5′-CTAGTCTAGATCAGC CGATCACGCCGCCATC-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 OD_{600} of approximately 0.5, chilled on ice for 20 min and centrifuged at $4000 \times g$ 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′-CCGGCAGAGATTCTGCT AGCA-3′, flank-R:5′-AGGGCTTAACCCGGATCGTG CG-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-EU669 020, 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

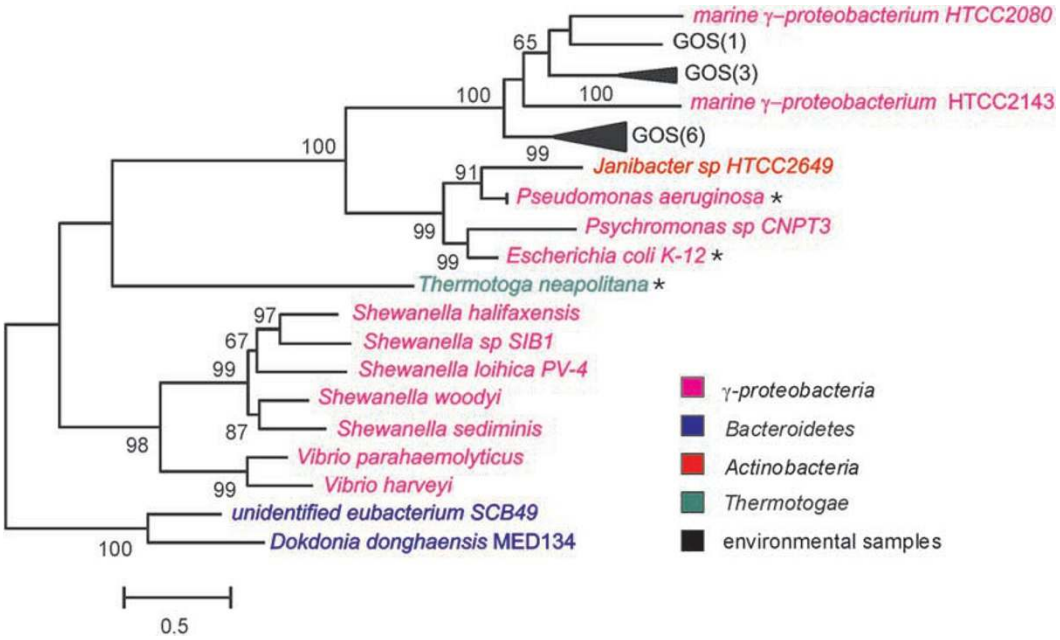

Figure 1 Phylogenetic relationships of *phoA* homologs from marine bacterial isolates and marine metagenomic libraries (GOS). Sequences were retrieved from NCBI by BLASTX using *E. coli phoA* (*e*-value $<10^{-35}$). Non-marine isolates with confirmed phosphatase activity were included in the analyses 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

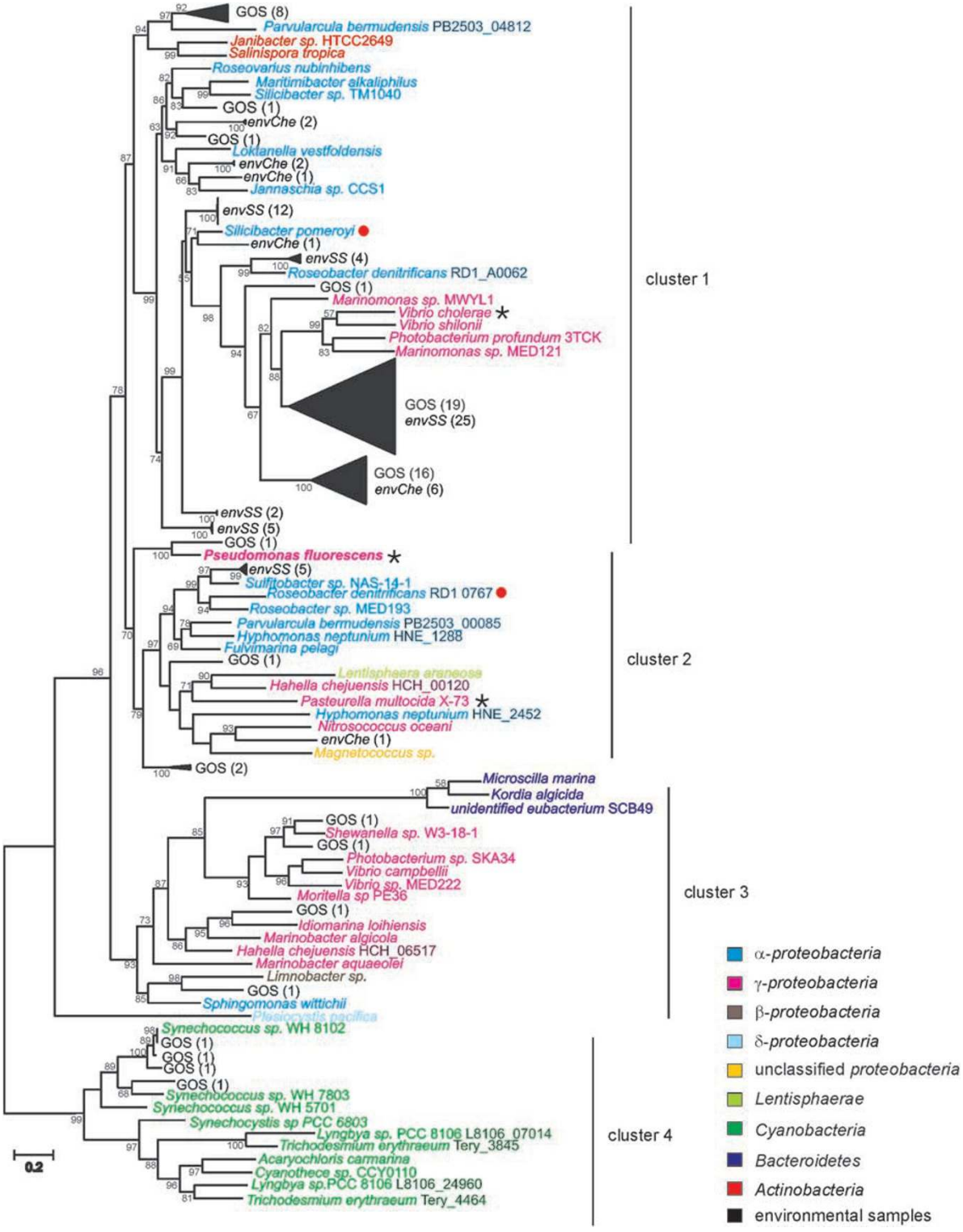

Figure 2 Phylogenetic relationships of representative *phoX* homologs from marine bacterial isolates and marine metagenomic libraries (GOS). Sequences were retrieved from NCBI by BLASTX using *V. cholerae phoX* (*e*-value $<10^{-35}$). Non-marine isolates with confirmed phosphatase activity were included 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.

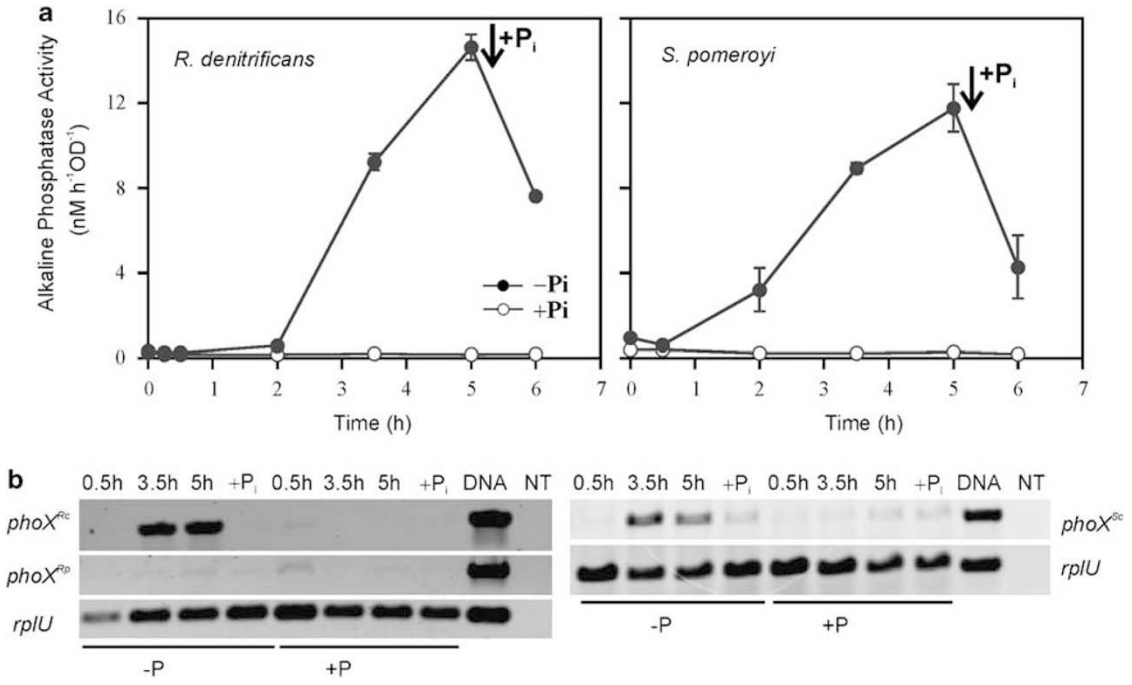

Figure 3 Time course of P-starvation response in marine isolates. (**a**) Induction of alkaline phosphatase activity and (**b**) expression of *phoX* in *R. denitrificans* and *S. pomeroyi* cells resuspended in medium with no added P ($-P_i$) compared with cells resuspended in P_i-replete medium ($+P_i$). Arrows indicate when phosphate was added back to the medium. $phoX^{Rc}$ (RD1_0767): *R. denitrificans* chromosome *phoX*; $phoX^{Rp}$ (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.

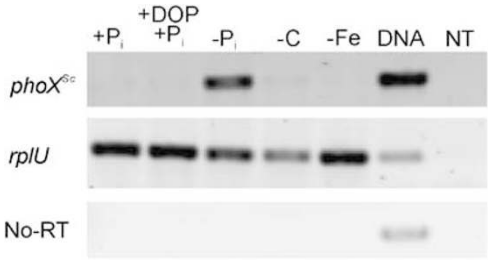

Figure 4 Expression of *phoX* in *S. pomeroyi* cells resuspended in P_i-replete medium ($+P_i$), P_i-replete medium supplemented with G6P as an organic P source ($+$DOP), P-free medium ($-P_i$), C-free medium (no glucose added, $-$C), Fe-free medium ($-$Fe). DNA: DNA control. NT: no template control. *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

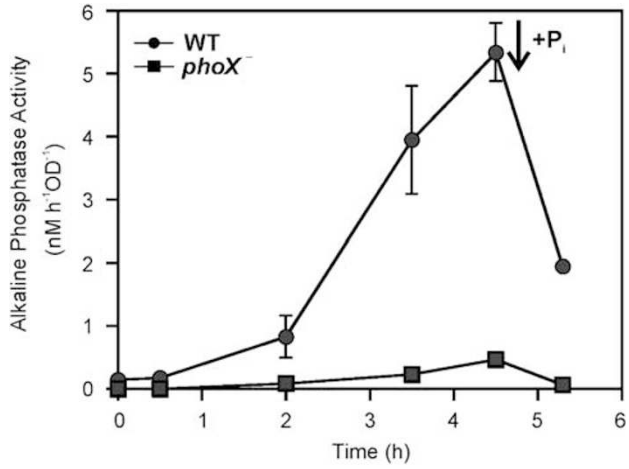

Figure 5 Time course of alkaline phosphatase activity in *Silicibacter pomeroyi* wild type (WT) and the *phoX*$^{-}$ mutant (*phoX*$^{-}$). The arrow indicates when inorganic phosphate (P_i) was added back to the medium. Errors bars represent the standard deviation of three replicates.

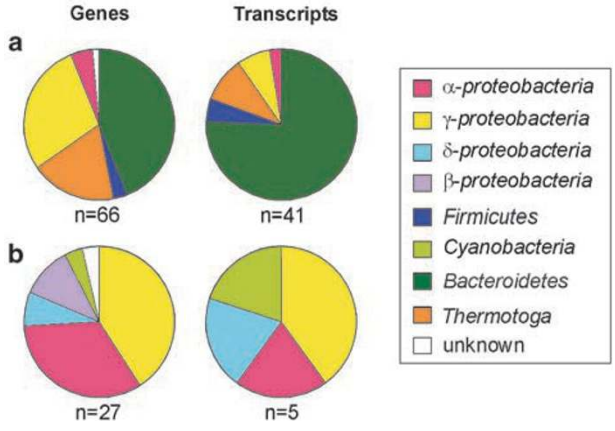

Figure 6 Heterogeneous P_i-stress response in marine bacteria. Putative taxonomic affiliation of phosphatase genes and transcripts in a coastal mesocosm experiment (Gilbert *et al*, 2008) (**a**) *phoA,* (**b**) *phoX* (see Supplementary Tables 3 and 4 for more information).

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)

	phoA	*phoX*	*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 Ca^{2+} instead of Zn^{2+} for PhoX catalytic activity has probably been a major factor in its selection over PhoA in marine environments, where Zn^{2+} often occurs at subnanomolar concentrations (Lohan *et al.*, 2002). Indeed, replacement of Zn^{2+} by other elements has also been observed in other metalloenzymes as an adaptative 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.

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