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Phosphate insensitive aminophosphonate mineralisation within oceanic nutrient cycles

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Abstract

Many areas of the ocean are nutrient-poor yet support large microbial populations, leading to intense competition for and recycling of nutrients. Organic phosphonates are frequently found in marine waters, but require specialist enzymes for catabolism. Previous studies have shown that the genes which encode these enzymes in marine systems are under Pho regulon control and so are repressed by inorganic phosphate. This has led to the conclusion that phosphonates are recalcitrant in much of the ocean where phosphorus is not limiting despite the degradative genes being common throughout the marine environment. Here we challenge this paradigm and show, for the first time, that bacteria isolated from marine samples have the ability to mineralise 2-aminoethylphosphonate, the most common biogenic marine aminophosphonate, via substrate-inducible gene regulation rather than via Pho-regulated metabolism. Substrate-inducible, Pho-independent 2-aminoethylphosphonate catabolism therefore

represents a previously unrecognised component of the oceanic carbon, nitrogen and phosphorus cycles.

Introduction

Inorganic nitrogen and phosphorus levels in the oceans are often limiting for microbial growth, leading to a heavy reliance on organic nutrient consumption to obtain these elements (Moore *et al.*, 2013). One class of organic phosphorus compounds is the phosphonates, molecules with a direct carbon-phosphorus bond: approximately 25% of high molecular weight dissolved organic phosphorus in the oceans is in phosphonate form (Clark *et al.*, 1999; Kolowith *et al.*, 2001), while up to 16% of the phosphorus taken up by marine plankton is converted to reduced phosphorus compounds including phosphonates (Van Mooy *et al.*, 2015). These studies demonstrate that large quantities of reduced phosphorus compounds - more than the annual pre-anthropogenic riverine input of phosphorus to the oceans - are being continuously and rapidly synthesised globally in the oceans, although the functions of these compounds are currently unknown. Many roles have been proposed for biogenic phosphonates including facilitating bacterial parasitism, altering the permeability of membranes, as a signalling molecule and as a way of increasing the resistance of membranes to phospholipases (Mukhamedova and Glushenkova, 2000; Steiner *et al.*, 1973). Additionally, when Van Mooy *et al.* (2015) noted that a *Trichodesmium sp.* released the majority of newly synthesised reduced phosphorus compounds into the environment they suggested that this may be a way of passing nutrients to epibiontic cells. However, to date there have been no studies which empirically demonstrate any of these functions, and therefore the ecological role of aminophosphonate compounds remains enigmatic. These molecules cannot be catabolised using organic phosphate degrading enzymes, but instead require phosphonate-specific enzyme systems for mineralisation. Previous studies using terrestrial microbes have suggested that these enzymes are, for the most part, under Pho regulon control and are therefore induced by inorganic phosphate starvation (White and

Metcalf, 2007). Phosphonates have thus only been considered in marine nutrient cycling models as sources of phosphorus, and even then only in phosphorus-limited waters, despite also containing carbon and frequently nitrogen. As a result these compounds are hypothesised to be recalcitrant in non-phosphate limited waters and the carbon, nitrogen and phosphorus within them removed from biologically cycled nutrient pools via sedimentation to the ocean floor. Consistent with this, phosphonate metabolism has only been previously demonstrated in marine microorganisms under phosphorus starvation conditions (Dyhrman *et al.*, 2006; Martínez *et al.*, 2013), and comparatively little consideration has been given to the possibility of organic phosphorus consumption as a carbon or nitrogen source in the presence of inorganic phosphate (Heath, 2005). However, metagenomic studies have shown that genes for phosphonate metabolism are common throughout the ocean even in regions with relatively high inorganic phosphate concentrations (Martinez *et al.*, 2010; Villarreal-Chiu *et al.*, 2012). In 2010 Martinez *et al.* showed that a *phnWX*-containing fosmid from a marine metagenomic library enabled an *E. coli* heterologous host to degrade 2AEP in the presence of inorganic phosphate when the transcriptional regulator was bypassed. This is consistent with previous studies which have shown that it is the repression of gene expression by inorganic phosphate which normally controls phosphonate degradation, rather than inhibition of the enzymes themselves (McGrath *et al.*, 2013). As a result an alternative regulation system similar to that previously observed in a terrestrial organism (Ternan and Quinn, 1998) could allow the genes already known to be present in the ocean to metabolise phosphonates in an inorganic phosphate-insensitive manner, rather than requiring novel degradation proteins. An NMR study of an anoxic basin found that phosphonates were preferentially metabolised relative to the more labile phosphate esters and, importantly, that this mineralisation occurred in the presence of >0.5 μM soluble reactive phosphorus, although no investigation into the organisms or specific phosphonate compounds involved was performed (Benitez-Nelson *et al.*, 2004). The Pho regulon is generally considered to be inactive at low-micromolar concentrations of inorganic phosphate (Wanner, 1996), suggesting that inorganic phosphate-insensitive phosphonate degradation does occur in

marine systems, analogous to that previously observed in a small number of terrestrial systems (Ternan and Quinn, 1998). Additionally, Clark *et al.* (1999) showed that the ratio of phosphonates to phosphate esters throughout the oceanic water column remains relatively stable despite the total quantity of organic phosphorus decreasing with depth, suggesting that phosphonates must be metabolised proportionately to phosphate esters even in deeper waters where inorganic phosphate tends to be more abundant.

The catabolism of aminophosphonates in non-phosphate-limited waters could be an important coping mechanism for marine microbes in waters of varying nutrient limitation, and provide an alternative nutrient niche for organisms which harbour such inorganic phosphate-insensitive phosphonate catabolism pathways. Large areas of the ocean surface are primarily nitrogen limited, and while phosphorus is expected to be the ultimate limiting nutrient in the oceans, nitrogen is more likely to be the proximate limiting nutrient (Tyrrell, 1999; Moore *et al.*, 2013). Long-term studies in the North Pacific Ocean suggest that oligotrophic environments shift back-and-forth between nitrogen and phosphorus limitation (Tyrrell, 1999; Karl *et al.*, 2001). The induction of phosphonate-degrading enzymes by conditions other than inorganic phosphate starvation would allow the use of aminophosphonates to supply microbial phosphorus, nitrogen and carbon requirements.

This metabolism would also provide a previously unrecognised route for the return of elements to actively cycled nutrient pools rather than loss via sedimentation. With oceanic primary productivity often constrained by nutrient supply (Karl *et al.*, 2001), the return of aminophosphonate derived carbon, nitrogen and phosphorus to more bioavailable forms via phosphate-insensitive catabolism would extend the contribution of microbial turnover to nutrient cycling beyond that previously described for Pho-regulated metabolism. Such phosphate-insensitive aminophosphonate degradation would also be expected to provide a competitive advantage to microbes in nutrient depleted environments. Given that phosphonate degradation models were developed using terrestrial microbes, we set out to examine the hypothesis that currently known phosphonate degradation systems exist in the marine environment under phosphate-insensitive regulation, allowing marine microbes to

consume phosphonates as a nitrogen source under high phosphate conditions which would previously have been expected to preclude this.

Materials and Methods

Reagents

1-hydroxy-2-aminoethylphosphonate was kindly provided by F. Hammerschmidt and K. Pallitsch, University of Vienna, Austria. Synthetic phosphonoacetaldehyde was kindly provided by the late H.B.F Dixon, Department of Biochemistry, Cambridge University, UK. Unless stated otherwise all other reagents were from Sigma Aldrich Co. (Poole, Dorset, UK).

Collection of oceanic surface water samples from around the island of Ireland

Environmental surface water samples (≤50 cm) were collected from coastal water bodies around the northern shores of Ireland and used to inoculate enrichment cultures. The sampling sites were: Portmuck Beach, Islandmagee, County Antrim; Mulroy Bay, County Donegal; Strangford Lough, County Down; and Kilkeel Harbour, County Down. Isolates from each water body were designated IMG, MRB, SGF or KKW respectively according to their place of origin. All samples were collected in sterile 50 or 15 mL centrifuge tubes (Sarstedt, Nümbrecht, Germany) or 30 mL sterile Universal tubes (Medline Industries Ltd, Cheshire, UK) and stored immediately at 4°C until they arrived at the lab, where they were inoculated into enrichment cultures without delay.

Inoculation of water samples into medium to enrich for and isolate 2AEP degrading bacteria

In order to promote the growth of microbes from the seawater samples which could use 2AEP as a nitrogen source enrichment cultures were performed in Minimal Marine Medium. This consisted of: 42.26 mM $MgSO_4.7H_2O$, 166.83 mM NaCl, 8.54 mM $CaCl_2.2H_2O$, 5.69 mM KCl and 50 mM HEPES sodium salt adjusted to pH 7.4. Ferric ammonium citrate at a

final concentration of 25 μM and 1 mL per litre each of trace elements (Krieg and Holt, 1981) and vitamin solution (Difco Laboratories, 1953) were added after autoclaving.

Standard nutrients for cultures consisted of 5 mM sodium succinate, 5 mM sodium acetate and 5 mM glucose as carbon sources, with 2 mM NH_4Cl and 1 mM KH_2PO_4 as nitrogen and phosphorus sources respectively.

Enrichment cultures were carried out in 30 mL sterile Universal tubes using 5 mL volumes of Minimal Marine Medium and synthetic, 99%-pure 2 mM 2-aminoethylphosphonate as the sole nitrogen source. 25 μL of the water samples were inoculated into separate Minimal Marine Medium volumes and incubated at 28°C with 100 revolutions per minute (RPM) shaking in a Sanyo ORBI-SAFE incubator (Sanyo Europe Ltd., Watford, UK). After 7 serial transfers morphologically different colonies were isolated from YTSSA plates (per litre 2 g yeast extract, 1.25 g vegetable peptone, 20 g Sigma Sea Salts, 1.5 % w/v agar) and 7 isolates which appeared to grow using 2-aminoethylphosphonate as the sole nitrogen source were selected for further study.

Growth studies of isolates with 2AEP as the sole nitrogen source

The ability of each isolate to use 2AEP as the sole nitrogen source was confirmed via growth studies. Colonies of each isolate were inoculated into 15 mL of YTSS medium (as per YTSSA but without agar) in a sterile 50 mL centrifuge tube and grown overnight at 28°C with 100 RPM shaking. The cells were centrifuged at 10 K x g for 15 minutes and washed twice in nutrient-free Minimal Marine Medium and used to make triplicate 5 mL cultures with and without 2 mM 2-aminoethylphosphonate as the sole nitrogen source in sterile 30 mL Universal tubes at a starting O.D. 650 nm of approximately 0.05, and incubated at 28°C/100 RPM. Optical density measurements at 650nm were taken at T0 and regular intervals using 200 μL samples with a Tecan GENios plate reader (Tecan Group Ltd., Männedorf, Switzerland), before being centrifuged and pellets and supernatants frozen separately. The phosphate concentration in the culture supernatants was measured using BIOMOL Green

(Enzo Life Sciences, Exeter, UK) while the pellets from T0 and the end of the exponential growth period were used for protein quantification using Bradford reagent, both according to the manufacturer's instructions.

Preparation of cell-free extracts from 2AEP-degrading bacteria

Cell-free extracts of each isolate were prepared for use in subsequent enzyme assays. A 250 mL culture of each isolate in YTSS was grown overnight at 28°C/100 RPM. The cells were centrifuged at 10 K x g for 10 minutes, washed twice in nutrient-free Minimal Marine Medium, re-suspended in 250 mL Minimal Marine Medium with 2 mM 2-aminoethylphosphonate as the sole nitrogen source and incubated at 28°C/100 RPM for 16 hours. The cells were then centrifuged and the pellet frozen at -80°C. After a minimum of two hours the pellet was defrosted on ice for 15 minutes, re-suspended in 3 mL 50 mM MOPS buffer (pH 7.5) and sonicated in an MSE Soniprep 150 Plus sonicator (MSE UK Ltd., London, UK) for 10 bursts of 20 seconds at 16.5 μm amplitude on ice with 20 second rests in between. The suspension was centrifuged at 18 K x g for 30 minutes at 4°C and the supernatant passed through a 0.22 μm syringe filter (EMD Millipore Corp., MA, USA). The extract was supplemented with 20% w/v glycerol, the protein concentration quantified using Bradford reagent and then stored at -20°C.

Enzyme assays to quantify phosphonate degrading activities in cell-free extracts

The PhnA, X and Z-like activities in cell-free extracts prepared from each isolate were examined via enzyme assays. Assay components for "PhnA"-type assays were 50 mM MOPS buffer (pH 7.5), 1 mM $ZnSO_4$ and 10 mM phosphonoacetic acid. "PhnX"-type assays contained 50 mM Tris buffer (pH 8.5), 5 mM $MgCl_2$ and 10 mM phosphonoacetaldehyde. 1-hydroxy-2-aminoethylphosphonate -degrading activity was determined using a modified version of the method described by McSorley et al. (2012) and contained 50 mM MOPS buffer (pH 7.5), 2 mM α-ketoglutarate, 0.2 mM sodium ascorbate, 10 mM 1-hydroxy-2-

aminoethylphosphonate, 0.1 mM $Fe(NH_4)_2(SO_4)_2$ and 0.1 mM $CaCl_2$. Although McSorley *et al.* do not state that PhnZ has a requirement for calcium ions they observed that PhnZ appeared to bind half as much calcium as iron, and so the $CaCl_2$ was included as a precaution.

250 μL of reaction mix was prepared for each assay replicate. Triplicate samples were prepared for each assay as well as triplicate extract-free and substrate-free controls. Addition of isolate protein extract (final concentration 0.5 mg protein mL^{-1}) was always performed last and the tube briefly vortexed. A 125 μL T0 sample was immediately removed and, as PhnA, X and Z are all metalloenzymes, the reaction in this sample was stopped by mixing with 25 μL 100 mM EDTA, vortexing and freezing. The remaining volume was incubated for 16 hrs in a Sanyo ORBI-SAFE incubator at 28°C and 100 RPM. At the end of the incubation period tubes were stopped as per the T0 samples.

Phosphate release was quantified using BIOMOL Green. Acetic acid quantification was carried out using an Acetic Acid Kit (Acetate Kinase Manual Format; Megazyme International Ireland, Co. Wicklow, Ireland) via the manufacturer's recommended protocol for a microplate assay.

Determination of the nutrient conditions which induce the phosphonoacetic acid-cleaving protein in cell-free extracts of a 2AEP-degrading isolate by zymography

A 500 mL YTSS culture of isolate IMG22 was incubated at 28°C/150 RPM overnight. The cells were centrifuged at 10 K x g for 10 minutes and washed twice in nutrient-free Minimal Marine Medium before being resuspended in 6x50 mL volumes of Minimal Marine Medium with no carbon, no nitrogen, no phosphorus, with 2 mM 2-aminoethylphosphonate as the sole nitrogen source, with all standard nutrients (replete) or with all nutrients and 2 mM 2-aminoethylphosphonate. Cultures were incubated and processed as described above. Protein extracts were concentrated in Amicon Ultra centrifugal filters (Merck Chemicals Ltd., East Midlands, UK; 3 kDa nominal molecular weight limit) as per the manufacturers

recommendations until a volume of <0.5 mL remained, at which point all extracts were made up to 0.5 mL using 50 mM pH 7.5 MOPS buffer with 20% w/v glycerol. The extracts were run on duplicate Novex WedgeWell 8-16% Tris-Glycine Mini 1 mm native-PAGE gels in a Novex XCell 2 mini electrophoresis system (Life Technologies Ltd., Paisley, UK) using the protocol for native PAGE gels described by the manufacturer. Lane 1 contained Novex NativeMark unstained protein standards (Life Technologies Ltd., Paisley, UK), and the protein extracts were loaded into the other lanes in equal (200 μg) quantities. The gels were run at 125 V for 80 minutes and then removed from the cassettes as recommended by the manufacturer. Coomassie Brilliant Blue staining and destaining were carried out on one gel as described by the manufacturer.

The second gel was used to assay phosphonoacetic acid cleaving activity via zymography. The gel was covered in 50 mM pH 7.5 MOPS buffer, 1 mM $ZnSO_4$ and 10 mM phosphonoacetic acid and incubated at 30 °C for 2 hours. The PhnA buffer was removed, the gel briefly rinsed in water and then covered with Fiske and Subbarow reagent (800 mg Fiske and Subbarow reducer in 5 mL water combined with 15 mL 21.5 mM ammonium molybdate tetrahydrate in 2.5 M H_2SO_4) to stain for phosphate release. After incubation at room temperature for 20 minutes the gel was briefly washed in water and then imaged.

Identification of the 2AEP degrading isolates by 16S rDNA sequencing

The sequence of the gene encoding 16S rRNA in each isolate was obtained using the 63f and 1387r universal primers described by Marchesi et al. (1998). PCR mixtures (25 μL) consisted of 1x Sigma PCR-buffer without $MgCl_2$, 2.5 mM $MgCl_2$, 3.5 pmol 63f primer, 7 pmol 1387r primer, 200 μM dATP, dCTP, dGTP and dTTP each (Life Technologies Ltd., Paisley, UK), 0.25 units of Taq DNA polymerase and a 1 μL tip of colony from a growing YTSSA plate of the isolate. The PCR was run on an Eppendorf Mastercycler Pro S PCR machine (Eppendorf UK Limited, Stevenage, UK) programmed for an initial 95°C step for 4 minutes, and then following the protocol described by Marchesi et al. (1998).

The PCR products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega UK, Southampton, England) according to the manufacturer's instructions and Sanger sequenced by GATC Biotech AG (Cologne, Germany) before being compared to the NCBI non-redundant nucleotide database excluding uncultured/environmental sample sequences (Altschul *et al.*, 1990).

Statistics

In all quantitative Figures the data are the mean of three biological replicates and the error bars are presented as the Standard Error of the Mean. *p*-values were calculated using a Student's t-Test to a significance level of 95%.

Results

Inorganic phosphate starvation is not required for growth using 2-aminoethylphosphonate as a nitrogen source

Although analytical limitations have hindered attempts to identify precisely which phosphonates are present in ocean water (Young and Ingall, 2010) many studies have identified 2-aminoethylphosphonate (2AEP) in common marine organisms (Kittredge *et al.*, 1962; Kittredge and Hughes, 1964; Quin, 1965; Hori *et al.*, 1967; Quin and Quin, 2001) and it is believed to be the most abundant phosphonate in the oceans (Martinez *et al.*, 2010). To investigate the possibility of phosphate-insensitive 2AEP mineralisation, selective enrichment cultures of Atlantic marine surface water samples taken from around the island of Ireland were set up with 2 mM 2AEP provided as the sole nitrogen source. Seven bacterial isolates were obtained which showed significantly higher optical densities ($p \leq 0.001$ at the start of stationary phase, Supplementary Figure 1) and protein concentrations ($p \leq 0.002$ in all cases, Figure 1) when grown with 2 mM 2AEP as the sole nitrogen source than in non 2AEP-augmented controls, suggesting 2AEP catabolism despite the presence of

1 mM KH_2PO_4. In all seven isolates the metabolism of 2AEP was accompanied by the concomitant release of up to 86% of the aminophosphonate-derived phosphorus as inorganic phosphate (Supplementary Figure 1). No significant inorganic phosphate release was observed in control cultures in the absence of 2AEP.

The phylogenetic identity of each isolate was determined by sequencing the gene for 16S rRNA and comparing it to the NCBI non-redundant database (Supplementary Table 1). Three were closely related to the genus *Falsirhodobacter*, and the remaining four were members of the genera *Rhodobacter*, *Sphingorhabdus*, *Terasakiella* or *Stappia*. All of these isolates were therefore α-Proteobacteria, consistent with published data showing that marine α-Proteobacteria are a major source of phosphonate catabolism genes, and in particular of the *phnA* gene, in the ocean (Villarreal-Chiu *et al.*, 2012; Shilova *et al.*, 2014).

The concentration of inorganic phosphate supplied in these experiments (1 mM) was more than two orders of magnitude greater than the peak concentration of soluble reactive phosphorus frequently found throughout the oceans (approximately 3 μM: Karl and Björkman, 2002; Paytan and McLaughlin, 2007). The point at which microorganisms become phosphorus-starved is unclear: while *Escherichia coli* begins to express phosphorus starvation genes at approximately 4 μM inorganic phosphate (Wanner, 1996), studies of phytoplankton communities in the Red Sea have shown that 0.1 μM soluble reactive phosphorus induces only low levels of phosphorus starvation-inducible protein production (Mackey *et al.*, 2007). This is presumably due to communities from oligotrophic environments being more adapted to survival in low nutrient conditions than terrestrial or lab-adapted enteric bacteria such as *E. coli*.

Only the products of the PhnA enzyme are detected in cell-free extract assays

A number of microbial phosphonate degradation pathways, three of which specifically catabolise 2AEP, are known to exist (Supplementary Figure 2). Cell-free assays of the

300 phosphonate-cleavage enzymes involved, termed PhnA, PhnX and PhnZ, have been
301 performed in previous studies and act on the terminal substrates of different degradative
302 pathways: phosphonoacetic acid as part of the PhnWYA pathway, phosphonoacetaldehyde
303 as part of the PhnWX pathway, and 1-hydroxy-2-aminoethylphosphonic acid as part of the
304 PhnYZ pathway (Supplementary Figure 2: Cooley et al., 2011; Borisova et al., 2011;
305 McSorley et al., 2012; Ternan and Quinn, 1998). While the non-specific C-P lyase pathway
306 has also been shown to break down 2AEP, *in-vitro* activity is lost when the cells are lysed
307 (Metcalf and Wanner, 1993), as in this cell-extract based analysis.
308 Cell-free extracts of each of the 7 isolates grown on 2AEP as a nitrogen source were
309 prepared and phosphonate bond cleaving activity was assayed by measuring the amount of
310 inorganic phosphate and organic product liberated from the terminal intermediate of each
311 pathway. Only incubation of cell extracts with phosphonoacetic acid (10 mM) resulted in the
312 release of organophosphonate derived inorganic phosphate (Figure 2). Phosphonate
313 cleavage was confirmed by the detection of equimolar concentrations of acetic acid ($p \geq 0.063$
314 in all cases), the organic product of phosphonoacetic acid mineralisation (Figure 3). The
315 inorganic phosphate insensitive mineralisation of 2AEP in these isolates is therefore
316 consistent with the phosphonoacetate hydrolase (PhnA) pathway, where 2AEP is
317 sequentially converted to phosphonoacetaldehyde, phosphonoacetate and then acetic acid
318 and inorganic phosphate by the PhnW, Y and A enzymes respectively (Supplementary
319 Figure 2: Cooley et al., 2011). The *phnA* gene has previously been shown to be the most
320 abundant phosphonate cleavage gene in marine metagenomes, with up to 11.2% of
321 expected bacterial genome equivalents containing a copy (Villarreal-Chiu *et al.*, 2012).
322 While a single instance of a phosphate-insensitive PhnA enzyme responsible for the direct
323 catabolism of phosphonoacetate has been reported (Kulakova *et al.* 2001), no previous
324 studies have shown the existence of a complete PhnWYA pathway for 2AEP degradation
325 under phosphate-insensitive regulation.
326

The phosphonoacetic acid-cleaving enzyme is induced by the presence of 2AEP, not by starvation

To investigate the induction of the phosphonoacetic acid-degrading activity, isolate IMG22 – an isolate closely related to the genus *Falsirhodobacter* - was selected for further study as it gave the highest activity levels in the initial experiments. A single pre-culture of IMG22 was split between 6 subcultures and each exposed to a different nutrient regime: one culture was supplied with all macronutrients (5 mM glucose, succinate and acetate each as carbon sources, 2 mM NH_4Cl as a nitrogen source and 1 mM KH_2PO_4 as a phosphorus source); three cultures lacked any carbon or nitrogen or phosphorus sources respectively, but were otherwise replete; one was replete but with the 2 mM NH_4Cl replaced by 2 mM 2AEP; and one culture was replete and also contained 2 mM 2AEP as an additional nitrogen source. After 16 hours of incubation proteins were extracted and a PhnA zymogram was prepared and stained for inorganic phosphate release (McGrath and Quinn, 1995) (Figure 4).

Only protein extracts from cells grown in the presence of 2AEP showed a zone of phosphate release in the zymogram after incubation in the presence of 10 mM phosphonoacetic acid, indicating phosphonoacetic acid cleavage and thus PhnA-like activity, regardless of the other nutrient conditions those cells were exposed to. No activity was observed in phosphorus, carbon or nitrogen starved samples (Figure 4). Phosphonoacetate cleavage activity correlated with the position of a 66 kDa protein standard (Supplementary Figure 3), consistent with the size of previously characterised PhnA proteins (McGrath and Quinn, 1995).

Discussion

The synthesis of 2AEP and distribution of its catabolic genes appears to be almost ubiquitous in the marine environment (Karl, 2014). This, paired with data showing that many

areas of the ocean are nitrogen and/or phosphorus limited, would suggest that 2AEP could be used as an organic nitrogen or organic phosphorus source by microorganisms which harbour aminophosphonate degradative gene clusters. Indeed, Karl (2014) notes that common phosphonate degradation genes are present in distantly related bacterial groups, likely the result of horizontal gene transfer driven by strong selective pressures for nutrient acquisition systems. Despite this, no microorganism has been shown to degrade 2AEP via any pathway in inorganic phosphate-replete marine waters even though a single instance of PhnWX-mediated phosphate-insensitive 2AEP catabolism has been described in a terrestrial microbe (Ternan and Quinn, 1998). If such activity were present in marine organisms this would further extend the potential of aminophosphonates to contribute to the marine carbon, nitrogen and phosphorus cycles.

To our knowledge this study is the first demonstration of growth via inorganic phosphate-insensitive 2AEP degradation by any pathway in aquatic bacteria, and suggests that aminophosphonate compounds are metabolically and biogeochemically active over a wider spectrum of inorganic phosphate concentrations than previously thought. Additionally, this is the first demonstration of inorganic phosphate-insensitive 2AEP catabolism by a PhnWYA-like pathway in any organism: the pathway has previously only been demonstrated as a method for obtaining phosphorus from 2AEP under inorganic phosphate starvation conditions (Borisova *et al.*, 2011; Cooley *et al.*, 2011). This metabolism appeared to be relatively rapid, with the majority of phosphate release from 2AEP completed within 24 hours (Supplementary Figure 1). This is mirrored by the rapid marine phosphonate synthesis demonstrated by Van Mooy *et al.* (2015), and is consistent with their suggestion that phosphorus is quickly cycled between reduced and oxidised forms in the ocean. The exact concentration of 2AEP in bulk ocean water – and the concentration necessary to induce phosphate-insensitive catabolism – is not known, but previous studies have identified many 2AEP-synthesising marine organisms (e.g. Quin and Quin, 2001) and NMR studies have shown that phosphonates are continuously mineralised throughout the water column (Clark *et al.* 1999), in line with the almost ubiquitous distribution of 2AEP catabolic genes in marine

systems (Villarreal-Chiu *et al.*, 2012). While lab-cultivatable microbes may not necessarily be representative of environmental communities as a whole, these isolates clearly demonstrate that phosphate insensitive aminophosphonate metabolism is present in the oceans.

The ability of some organisms to cycle aminophosphonates regardless of local inorganic phosphate concentrations would accelerate the remineralisation of the carbon, phosphorus and nitrogen contained within them and return these to nutrient pools which are more available to non-phosphonate degrading organisms. Some studies suggest that marine nitrogen limitation is more prevalent than marine phosphorus limitation (e.g. Moore et al., 2013), and so the catabolism of 2AEP as a nitrogen source may be more relevant to microbial function, nutrient turnover and biogeochemical cycling than the catabolism of 2AEP (or other aminophosphonates) as a phosphorus source. Furthermore, aminophosphonate nitrogen supply may explain the previously noted NMR study which showed preferential phosphonate consumption relative to phosphate esters under relatively high-phosphorus conditions (Benitez-Nelson *et al.*, 2004).

It has been suggested that our understanding of the role of organic phosphorus in aquatic ecosystems is unduly focussed on fuelling phosphorus-limited planktonic growth, something termed the “phosphorus-limited planktonic view” (Heath, 2005). This study, which demonstrated that some marine bacteria can catabolise the aminophosphonate 2AEP regardless of cellular phosphorus status, would suggest a wider role for phosphonates within the marine nutrient pool than is currently recognised. Our finding of inorganic phosphate-insensitive aminophosphonate catabolism parallels several studies which found relatively high levels of alkaline phosphatase activity in deep waters where marine inorganic phosphate concentrations tend to be highest (Koike and Nagata, 1997; Baltar *et al.*, 2009; Hoppe and Ullrich, 1999). This growing body of research showing that organic phosphorus catabolism is not always controlled by environmental phosphate concentrations suggests a wider role than that previously ascribed by the “phosphorus limited planktonic view”, one

encompassing the supply and recycling of bioavailable carbon and nitrogen in addition to phosphorus to the marine ecosystem.

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Conflict of interest

The authors declare no competing financial interests.

Supplementary information

Supplementary information is available at *The ISME Journal*'s website

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Figure Legends

Figure 1: Protein concentrations of isolate cultures with (white bars) and without (grey bars) 2mM 2AEP as the sole nitrogen source at the start of growth and at the approximate beginning of stationary phase. Y axis shows protein concentration in μg per mL culture, X axis show time in hours, isolate identifiers are shown in the top left of each subfigure. Data are averages of biological triplicates; error bars show standard error of the mean.

Figure 2: Inorganic phosphate producing activities (in nmol released per minute per mg protein) of isolate protein extracts when given substrates for PhnA (phosphonoacetic acid, grey bars), PhnX (phosphonoacetaldehyde, chequered bars) or PhnZ (1-hydroxy-2-aminoethylphosphonic acid, white bars) enzymes. Data are averages of biological triplicates; error bars show standard error of the mean.

Figure 3: Inorganic phosphate and acetic acid producing activities (in nmol released per minute per mg protein) of isolate protein extracts. Inorganic phosphate is shown in the solid grey bars, and acetic acid in white bars. Data are averages of biological triplicates; error bars show standard error of the mean.

Figure 4: PhnA Zymogram of protein extracts from IMG22 cells exposed to different nutrient conditions. The dark spots in lanes 1 and 6 indicate phosphate release. Lane 1: cells in replete medium + 2 mM 2AEP, lane 2: cells in replete medium, lane 4: -phosphorus, lane 6: 2 mM 2AEP as sole nitrogen source, lane 7: -nitrogen, lane 9: -carbon, lane 10: Novex NativeMark protein standards, lanes 3, 5 and 8: no sample.

Figure 1

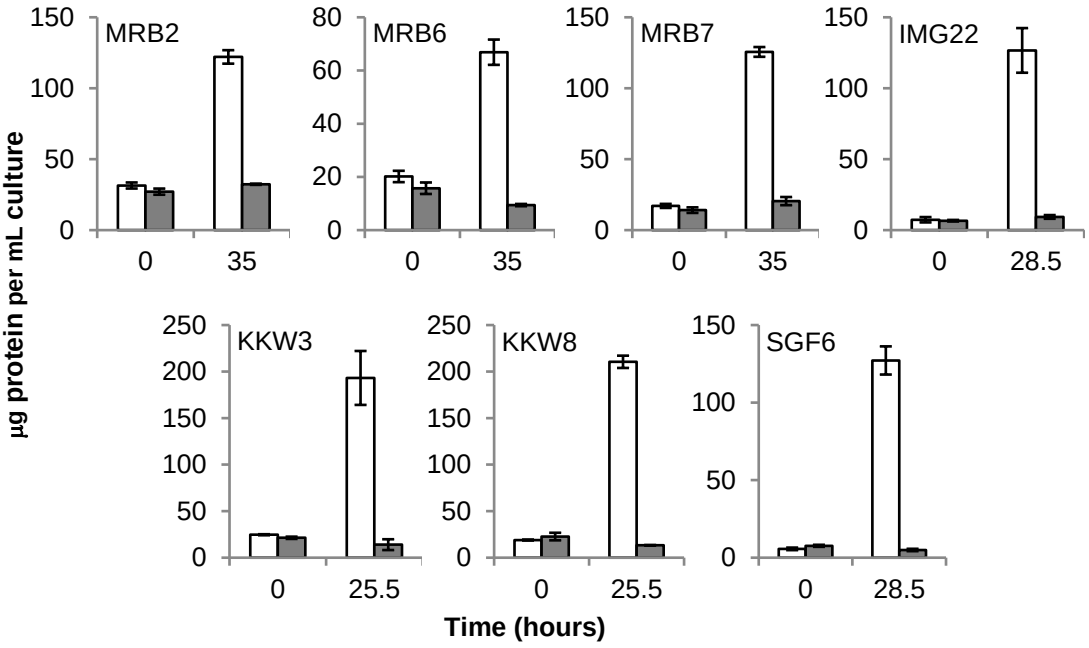

MRB2
MRB6
MRB7
IMG22
KKW3
KKW8
SGF6
µg protein per mL culture
Time (hours)

Figure 2

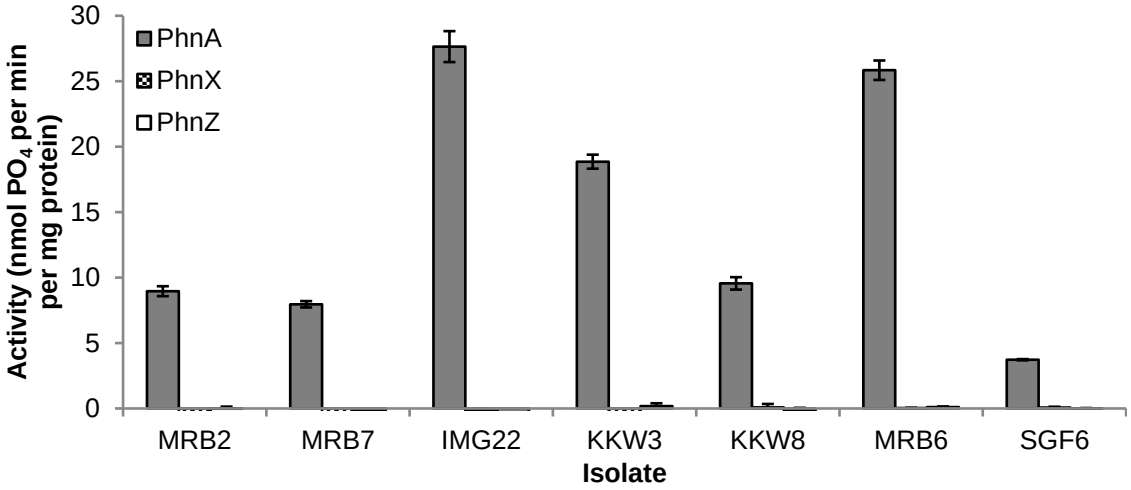

30
25
20
15
10
5
0
PhnA
PhnX
PhnZ
Activity (nmol PO4 per min per mg protein)
MRB2
MRB7
IMG22
KKW3
KKW8
MRB6
SGF6
Isolate

Figure 3

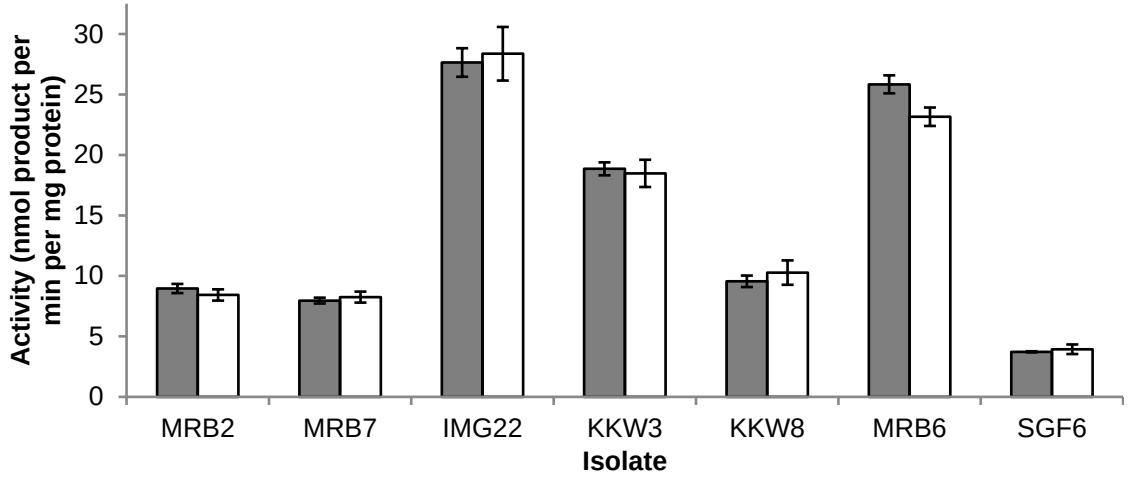
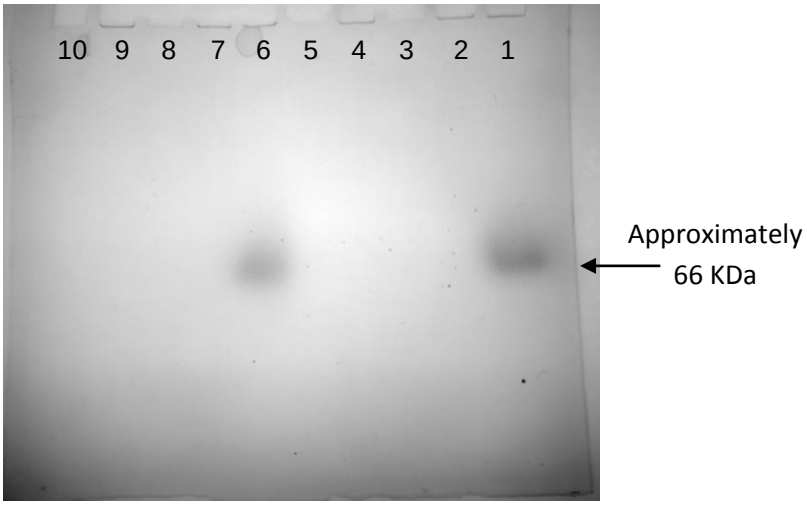
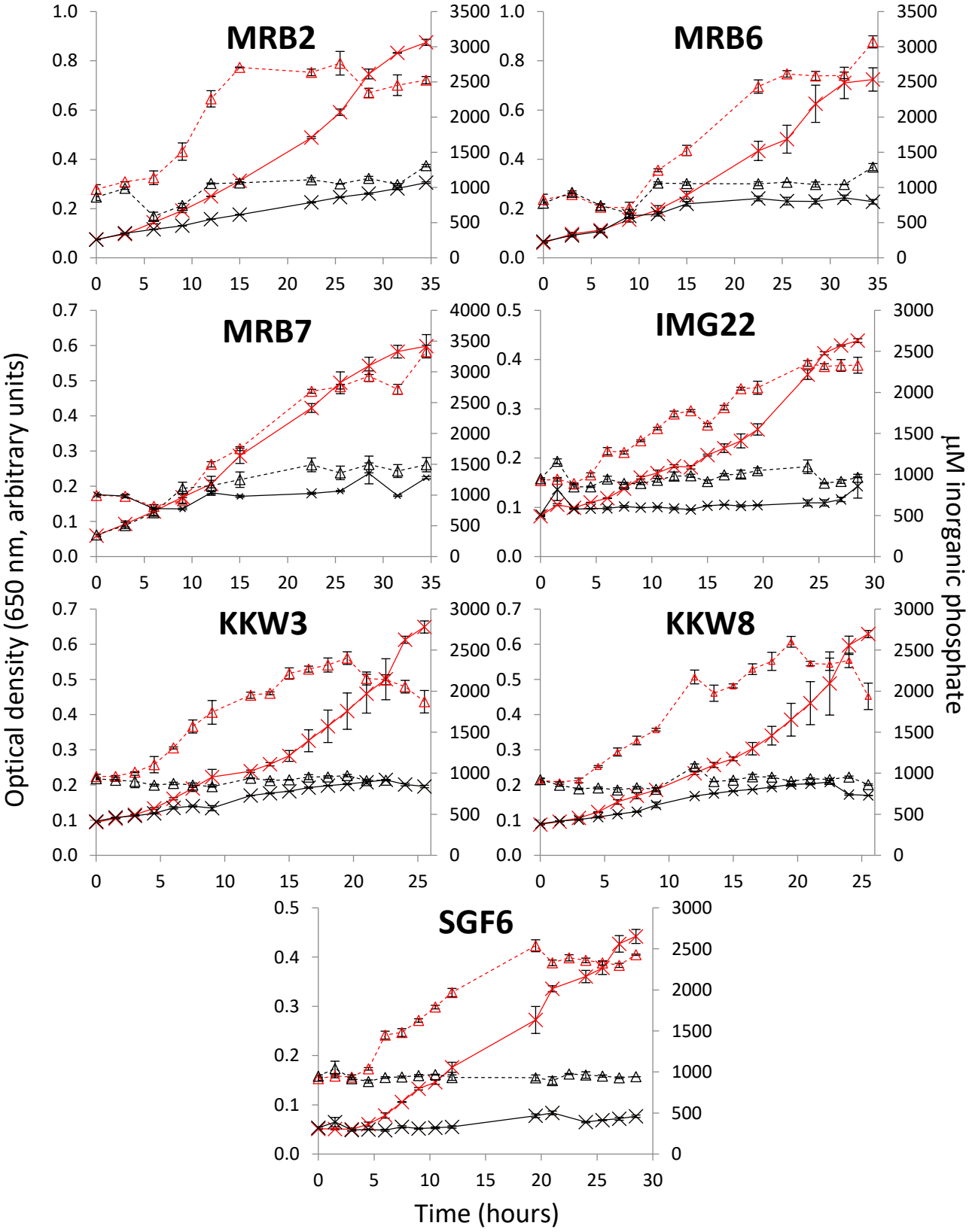

Activity (nmol product per min per mg protein)
30
25
20
15
10
5
0
MRB2
MRB7
IMG22
KKW3
KKW8
MRB6
SGF6
Isolate

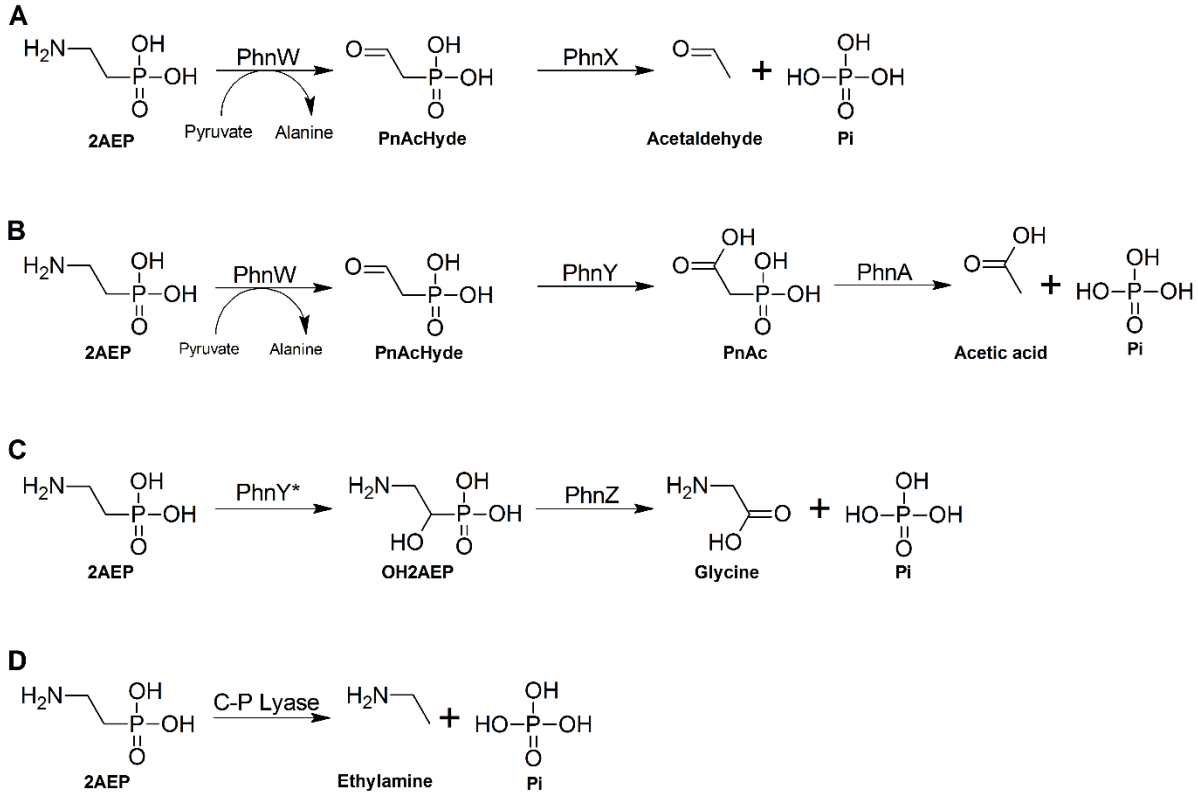
Figure 4



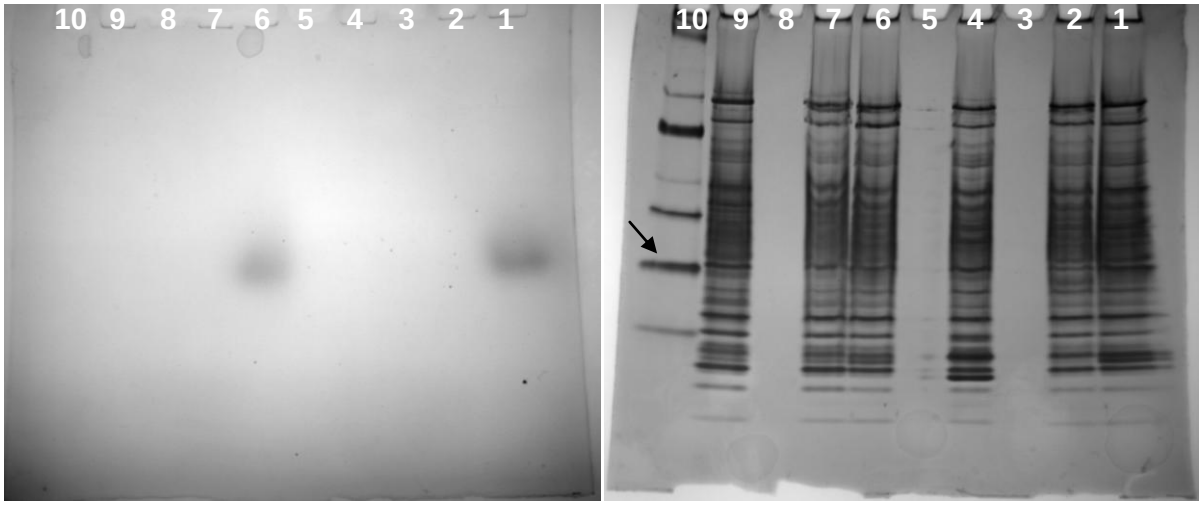


Supplementary Figure 1: Growth (solid lines) and medium Pi concentration (dashed lines) in isolates given 2 mM 2AEP as the sole nitrogen source (red) or with no nitrogen source (black). In all figures the horizontal axis is time in hours, left vertical axis is optical density at 650 nm in arbitrary units and right horizontal axis is medium inorganic phosphate concentration in µM. Isolate designations are

marked in bold font in the upper centre of each figure. All data points are the average of biological triplicates, all error bars are Standard Error of the Mean.



Supplementary Figure 2: 2-aminoethylphosphonic acid (2AEP) degradation pathways[1]. A) PhnW converts 2AEP to phosphonoacetaldehyde (PnAcHyde) and then PhnX cleaves the C-P bond to release acetaldehyde and orthophosphate (Pi). B) After conversion to PnAcHyde by PhnW, PhnY converts this to phosphonoacetic acid (PnAc) before PhnA acts on this to release acetic acid and Pi. C) 2AEP is oxidised to 1-hydroxy-2-aminoethylphosphonic acid (OH2AEP) by PhnY*, which is then processed to glycine and Pi by PhnZ. D) The C-P lyase complex will convert many phosphonates to the organic chain, in this case ethylamine, and Pi. Note that although Martinez *et al.*[2] named the 2AEP-oxidase protein in pathway C "PhnY" it bears no relation to the PhnY protein which had previously been identified in the PhnWYA pathway[3] and so it is identified as "PhnY*" here.



Supplementary Figure 3: Native polyacrylamide electrophoresis gels of IMG22 protein extracts from cells grown under different nutrient regimes. Right: gel stained with Coomassie Blue to indicate proteins. The black arrow indicates the 66 KDa standard band. Left: gel stained with Fiske and Subbarow reagent for phosphate after incubation in PhnA assay buffer. Lane 1: cells grown in replete medium + 2 mM 2AEP, lane 2: cells grown in replete medium, lane 3: empty, lane 4: phosphorus starved, lane 5: empty, lane 6: 2 mM 2AEP as the sole nitrogen source, lane 7: nitrogen starved, lane 8: empty, lane 9: carbon starved, lane 10: Novex NativeMark protein standards.

Supplementary Table 1: Most similar sequence in the NCBI database to the 16S rRNA gene of 2AEP-using isolates

Isolate	Most similar organism	Query	e Value	Identity
IMG22	*Falsirhodobacter halotolerans* strain DAS4B23	99%	0.0	99%
KKW3	*Rhodobacter* sp. CR07-62	99%	0.0	99%
KKW8	*Sphingorhabdus* sp. M41	100%	0.0	98%
MRB2	*Falsirhodobacter halotolerans* strain DAS4B23	99%	0.0	99%
MRB6	*Terasakiella pusilla* strain NBRC 13613	90%	0.0	98%
MRB7	*Falsirhodobacter halotolerans* strain DAS4B23	98%	0.0	98%
SGF6	*Stappia conradae* strain MIO	99%	0.0	99%

Supplementary Information references:

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