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Bacterial utilization of creatine in seawater

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ABSTRACT: Dissolved organic nitrogen (DON) is recognized as an important component of the marine carbon (C) and nitrogen (N) cycles. Creatine, a component of the dissolved free amino acid (DFAA) pool, is a known byproduct of metazoan metabolism, and genetic evidence suggests that some phytoplankton may also have the ability to produce creatine. We hypothesized that creatine utilization by marine bacteria is more widespread than commonly assumed. The diatom *Thalassiosira pseudonana*, for which genome analysis had indicated the potential for creatine synthesis, was used to verify the presence of creatine via liquid chromatography/mass spectrometry (LC/MS) analysis. The phylogenetic breadth of creatine-utilizing bacteria and protists was investigated via a bioinformatics approach. Uncharacterized creatinases found in the genomes of *Roseobacter denitrificans* Och114 and *Roseobacter litoralis* Och149 were sub-cloned, hexa-histidine tagged, and expressed in *E. coli* to confirm their functional annotation. Enzymatic activity assays indicated optima at pH 8.4 and 35°C with K_m values of 25 to 27 mM. A field experiment was conducted in the equatorial Pacific, where creatine concentrations were found to range between 19 and 171 nmol N l^{-1}, with higher concentrations at the surface than at the deep chlorophyll maximum (DCM). ^{15}N-tracer techniques were used to measure creatine uptake rates, which were in the range of 0.08 to 0.66 nmol N l^{-1} h^{-1}, and were higher in surface waters than at depth. Overall, these data support the idea that phytoplankton are a potential source of creatine to marine bacteria, and that creatine utilization by marine bacteria might account for a measurable fraction of DFAA turnover in the oceans.

KEY WORDS: Creatine · DON · Nitrogen · Marine nitrogen cycle · Diatoms · Marine bacteria

INTRODUCTION

Dissolved organic nitrogen (DON) is the largest pool of fixed N in much of the surface oceans (Bronk 2002). Traditionally, the DON pool has received relatively less attention than dissolved inorganic nitrogen (DIN) due to its perceived refractory nature, though it is now understood that DON is in fact a key link between the marine nitrogen (N) and carbon (C) cycles. In aquatic systems, ^{15}N tracer studies have demonstrated that rates of both DON production (e.g. Bronk & Glibert 1991, Bronk et al. 1998, Bronk & Ward 2005) and consumption (Bronk & Glibert 1993) are high, revealing that DON turnover can be significant on short and sub-seasonal time scales (Berman & Bronk 2003). The chemical composition of DON therefore represents a gradient of refractory to relatively more labile compounds (Sipler & Bronk 2015). More refractory compounds include complex organic matters such as humic and fulvic acids and a large range of poorly characterized higher molecular weight molecules. Labile fractions of DON include nucleic acids and their building blocks (e.g. purines and pyrimidines), urea, as well as dissolved free and com-

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bined amino acids (DFAAs and DCAAs). Remarkably, DFAA concentrations of up to 0.85 µmol N l^{-1} can be observed in non-estuarine marine environments, accounting for potentially as much as 3 to 12% of the DON pool in these systems (Sipler & Bronk 2015).

In terms of lability, DON compounds with high N:C ratios are likely to be preferred by both phyto- and bacterioplankton as N sources, as they likely require fewer enzymatic steps for the release of N in the form of ammonium (NH_4^+). Creatine ($C(NH_2)(NH)$-$N(CH_3)$-CH_2-CO_2H) has long been known as an important energy shuttle in metazoan muscle tissue where it serves to transfer energy from ATP in mitochondria to the sites of ATP utilization at the myofibrillar M line, the sarcoplasmic reticulum, and the plasma membrane (Wyss & Kaddurah-Daouk 2000). As a result, creatine is present in relatively high concentrations in metazoan excretions. It has been suggested that zooplankton do not excrete creatine in marine systems (Whitledge & Dugdale 1972), but published data in this context are scarce, and the availability of creatine in seawater is therefore generally ascribed to fish excretion. Peruvian anchoveta excretions, for example, contain on average 0.11 µg-atom (mg dry wt)$^{-1}$ d^{-1} creatine, which is comparable to NH_4^+ or urea excretions (0.13 and 0.08 µg-atom (mg dry wt)$^{-1}$ d^{-1}, respectively) (Whitledge & Dugdale 1972). A similar study focusing on anchovies reported creatine excretion rates of 3.4 µg-atom (g dry wt)$^{-1}$ h^{-1}, which similarly were in the range of NH_4^+ and urea excretions (4.5 and 3.3 µg-atom (g dry wt)$^{-1}$ h^{-1}) (Whitledge & Packard 1971).

More recently, the complete genome of *Thalassiosira pseudonana* revealed the genetic potential for a complete urea cycle, a property previously only attributed to metazoans (Armbrust et al. 2004, Allen et al. 2006). Arginine, formed as part of the urea cycle, is available for the production of the signaling molecule nitric oxide, the polyamine and proline precursor ornithine, or the high-energy molecule creatine phosphate (Allen et al. 2006). What role creatine and/or creatine phosphate might play in diatom physiology remains to be shown, and experimental evidence of creatine as a metabolite in diatom cells has not been reported. However, even small components of cellular N budgets in diatoms may have an impact on marine systems. Marine primary productivity amounts to roughly 50% of global total primary production (Falkowski et al. 1998, Field et al. 1998), and diatoms are thought to account for an estimated 40% of this activity (Nelson et al. 1995, Sarthou et al. 2005). Release of cellular metabolites due to cell death or leakage might therefore present a significant route for creatine release in marine waters beyond metazoan excretions.

Creatine metabolism liberates a number of N- and C-containing compounds that could support bacterial growth. These include urea via creatinases, NH_4^+ through the activity of urease, glycine via the enzyme sarcosine dehydrogenase, or dimethyl-glycine dehydrogenase and methylamine via the activity of sarcosine reductase (Wyss & Kaddurah-Daouk 2000). Over 40 yr ago, uptake of ^{14}C-labeled creatine by non-axenic diatom consortia was originally reported as a personal communication by Whitledge & Dugdale (1972), but was not substantiated by a subsequent publication. Whether marine bacteria can utilize creatine, at what rate this might occur, and what role creatine might play in overall DON budgets remains to be explored. Because of the way DON measurements are typically conducted, creatine is likely included in estimates of DFAAs (McCarthy & Bronk 2008), but the contribution of creatine to the DFAA pool and its turnover is not known.

The current study focused on investigating creatine as a potential component of the marine DON cycle, especially in light of a potential diatom-derived source of creatine. Our aims were to demonstrate the presence of creatine in *T. pseudonana* via targeted metabolite analysis, to lend support to the hypothesis that additional sources of creatine beyond metazoan excretions (notably phytoplankton metabolism) exist in marine systems. Secondly, we aimed to explore the diversity of potential creatine-utilizing bacteria and to show that genes commonly annotated as creatinases in the genomes of marine bacterial strains, notably *Roseobacter litoralis* OCh149 (DSM 6996) and *Roseobacter denitrificans* OCh114 (DSM7001), are not erroneously annotated and are in fact creatinase enzymes. Lastly, we aimed to demonstrate the presence and uptake rates of creatine in natural seawater samples derived from the equatorial Pacific.

MATERIALS AND METHODS

Targeted metabolite analysis

Thalassiosira pseudonana (CCMP1335) was obtained from the National Center for Marine Algae and Microbiota and was cultivated in F/2 medium prepared from natural, aged seawater under a 12 h light:12 h dark cycle at 18°C. Cells in exponential growth were collected by gentle filtration onto 0.2 µm polycarbonate membranes, and rinsed using

phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4; pH of 7.4) to remove residual creatine from seawater. Cells were placed onto an X,Y,Z–translation stage system and subjected to mass spectrometry (MS) analysis with the assistance of a microscope (Pan et al. 2014, 2016, Rao et al. 2016) coupled to a Thermo LTQ Orbitrap XL mass spectrometer. MS analyses were performed using the following parameters: mass resolution 100 000, ionization voltage 3.5 kV, sampling solvent methanol:water (1:1) with 0.05% formic acid. Protonated creatine ($[C_4H_9N_3O_2+H]^+$) was identified at a mass to charge ratio (*m/z*) = 132.0772 (theoretical *m/z* = 132.0773) using MS analysis. The detection of creatine was further confirmed by conducting collision induced dissociation (CID) tandem MS (MS^2) analysis of creatine ions on this mass spectrometer. The corresponding fragmentation mass spectrum was then compared to a creatine standard (Sigma Aldrich).

Bacterial cultures and medium

Roseobacter denitrificans Och114 (DSM 7001, ATCC 33942) and *R. litoralis* Och149 (DSM 6996, ATCC 49566) were obtained from the American Type Culture Collection (Manassas, VA) and propagated on Marine Agar/Broth 2216 (BD 279110). For growth tests (see Table 1), artificial seawater medium was prepared by adding 26 g l^{-1} Instant Ocean, 0.1 g l^{-1} ferric chloride, and 15 g l^{-1} ultrapure agarose. Glucose and creatine were added from sterile 1 M filtered stocks after autoclaving and cooling media to 65°C before pouring plates. After inoculation, plates were incubated at 18°C for 2 wk before evaluating cultures for growth.

DNA extraction and PCR

For DNA extraction, cultures were grown in Marine Broth 2216 and 1.0 ml of culture was spun at 13 000 × *g* for 2 min to pelletize cells. Cells were then re-suspended in 0.5 ml 1 × STE Buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA; pH 8) and 0.1 volumes of 20% sodium dodecyl sulphate (SDS). After inverting the tubes several times, cell lysates were extracted twice with TE saturated phenol, and once with chloroform. DNA was precipitated with 0.7 volumes isopropanol and 0.1 volumes 3 M sodium acetate (pH 5.2). After drying, DNA was re-suspended in 10 mM Tris-HCl pH 8 and stored at –20°C. The genes annotated as creatinase in the genomes of *R. denitrificans* Och114 and *R. litoralis* Och149 were PCR-amplified using primers that contain *Bam*HI and *Hind*III restriction sites for directional cloning. The forward primer for both reaction was as follows: Och149/114_BAMH1_F, 5′-AGA TCT GGA TCC ATG ACC ACT CAA TCC AAA AC-3′. Reverse primers were as follows: Och149_HIndIII_R, 5′-TCT AGA AAG CTT TCA GCC GAC GAC GTT AAA GC-3′ and Och114_HIndIII_R, 5′-TCT AGA AAG CTT CTA GCC GAC GAC GTT GAA GC-3′. Reactions were performed in 50 µl volumes using PCR Supermix (Invitrogen) containing 200 nM of each primer. Annealing temperatures were optimized by gradient PCR to 61°C and PCR cycling was performed as follows: 40 cycles of 95°C for 30 s, 61°C for 60 s, 72°C for 90 s, followed by a 10 min extension step at 72°C. PCR products were evaluated by gel electrophoresis to confirm the presence of a single product and cleaned using a QIAquick PCR purification kit (Qiagen). Products were then cloned by dual digestion using 20 µl reactions containing 10 U of *Bam*HI and *Hind*III and ligation into pQE80LC and pQE80LN vectors. Vectors (courtesy of Dr. Karr, University of Oklahoma) were modified to allow in-frame cloning of hexa-histidine tags at the N-terminal (pQE80LN) or C-terminal (pQE80LC) of the proteins (Karr 2010). Recombinant plasmids were then transformed into Top10F′ *E. coli* (Invitrogen), and clones were screened for inserts via colony PCR. Clones with inserts of the correct size were sequenced to confirm the absence of cloning artifacts (data not shown). One clone from each cloning experiment was chosen for downstream analysis (4 in total: hexa-histidine Tag on C or N terminal for creatinase from Och114 and Och149, respectively).

Protein purification

For protein purification, *E. coli* containing requisite plasmids were grown in LB medium containing 100 mg ml^{-1} ampicillin. Overnight cultures were inoculated into fresh medium (1:50 inoculum) and grown at 37°C in a shaking incubator (200 rpm) until an OD600 of 0.6 was reached. Expression was then induced with 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) and cells were incubated at 18°C overnight. Cells were harvested by centrifugation at 4000 × *g* for 20 min and cell pellets were stored at –80°C until protein purification. His-tagged proteins were purified using Ni-NTA agarose (Qiagen) as recommended by the manufacturer. In brief, cells were

re-suspended in 20 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole; pH 8.0), sonicated for 5 min (6 × 50 s intervals with 10 s pause at 250 W), and cell lysis was evaluated by microscopy. The lysate was then spun at 10 000 × *g* for 30 min at 4°C and 1 ml of Ni-NTA slurry was added the remaining supernatant. After washing the Ni-NTA agarose with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole; pH 8.0), elutions were performed by sequentially increasing the imidazole concentration to 75, 125, and 250 mM. All elutions, as well as insoluble protein, were evaluated via SDS-PAGE to assess protein purification. Protein was dialyzed into protein storage buffer (50 mM KH_2PO_4, 50% glycerol) using Slide-A-Lyzer MINI (Thermo Fisher) dialysis tubes (10KDa cutoff) to remove elution buffer components. Protein was quantified using Quick Start™ Bradford Protein Assay reagent (BIO-RAD) according to the manufacturer's instructions.

Enzyme assays

Creatinase catalyzes the conversion of creatine to sarcosine and urea (creatine + $H_2O \leftrightarrow$ sarcosine + urea). Creatinase activity was therefore assessed spectrophotometrically by quantifying dye formation via the condensation of urea and *p*-dimethylaminobenzaldehyde (DMAB) as outlined by Sigma Aldrich (www.sigmaaldrich.com/technical-documents/protocols/biology/assay-procedure-for-creatinase.html). For each assay, 5 mg of purified protein was suspended in 100 µl phosphate buffer (50 mM KH_2PO_4 buffer; pH 8.3) and 900 µl of 100 mM creatine (pH 7.5) were added. Reactions were incubated for 20 min and stopped by adding 2 ml DMAB reagent (1 g DMAB in 50 ml dimethyl sulfoxide [DMSO] and 7.5 ml 12.1 N HCl). Given that the temperature optima of the purified enzymes were at ~35°C (see 'Results'), all incubations were performed at 33°C and not at 37°C as is typically recommended for creatinase enzyme assays (Sigma Aldrich). Color production was quantified spectrophotometrically at 435 nm absorbance. The pH of the phosphate buffer used to suspend the enzyme was varied from 6 to 10 to assess its pH tolerance. Heated controls were generated by first heating purified protein to 80°C for 15 min and then adding the denatured protein to enzyme assays. The impact of major ions in seawater on enzymatic activity was assessed by quantifying the activity in the presence of NaCl, $MgSO_4$, $MgCl_2$, and Na_2SO_4 from 0 to 100 mM. Measurements of V_{max} and the Michaelis constant (K_m) were made in triplicate by varying the amount of creatine in the assay solution from 1 to 100 mM in 10 mM increments and analyzing the data via Lineweaver-Burk and Eadie-Hofstee plots (data not shown).

Field sampling

To assess the importance of creatine as an N substrate in the environment, we used ^{15}N-labeled creatine to measure creatine uptake. Samples were collected aboard the R/V 'New Horizon' between September and October 2014 on a cruise of opportunity in the tropical Pacific. Incubations were performed at 2 stations. Stn 1 was near Hawaii at 16.00° N, 156.37° W. Stn 2 was closer to the equator at 8.00° N, 153.50° W. Water was collected during daylight hours using 20 l Niskin bottles from both the surface and deep chlorophyll maximum (DCM). Incubations were conducted in 500 ml acid-cleaned polycarbonate bottles, which were placed under neutral density screening to simulate ambient light intensity. Bottles were incubated in deck-mounted flow-through incubators to keep temperatures near those of sampled seawater. Samples taken at Stn 1 were incubated for 6 h, while samples taken at Stn 2 were incubated for 24 h. After incubation, cells were filtered onto 0.2 µm silver filters, which were kept at −20°C until processing.

There are few data on N uptake rates for the equatorial Pacific in the literature. For comparison, we therefore present uptake rate data for a mixture of 16 amino acids as well as NH_4^+ and urea that were measured on a cruise of opportunity aboard the R/V 'Melville' in approximately the same region (Stn 3: 31° 00′ 00″ N, 159° 00′ 00″ W) in May 1999. The field methods used were the same as described above for Stns 1 and 2 with a few exceptions. Incubations were done in 1 l PETG bottles and lasted for 2 to 4 h. After incubation, cells were filtered onto pre-combusted (2 h at 450°C) GF/F filters (nominal pore size: 0.7 µm), and kept at −20°C until processing.

Creatine concentrations and uptake rates

Creatine concentrations in seawater samples were measured via liquid chromatography/mass spectrometry (LC/MS). Briefly, a known amount (final concentrations range between 1 and 1000 nmol N l^{-1}) of deuterated creatine ($C_4H_6D_3N_3O_2$; CDN Isotopes) was used as an internal standard and added to 0.2 µm filtered seawater. To minimize the influence

of salt on LC/MS experiments, desalting tubes (Supel™-Select SCX SPE Tube; Sigma-Aldrich) were used to remove salts from seawater. The desalted samples were then analyzed using a Thermo LTQ Orbitrap XL mass spectrometer equipped with a Nano-Electrospray Ionization (nano-ESI) source. Both naturally occurring and deuterated creatine can be simultaneously detected in this manner. The concentration of creatine in seawater was then derived from their relative ion intensities and the concentration of deuterated creatine. The detection limit of creatine in seawater was empirically determined to be ~20 nmol N l^{-1}, at which the measured signal-to-noise ratio of deuterated creatine was above 3.

Uptake rates were calculated as described previously (Bronk et al. 2014) using ambient creatine calculations determined via MS analysis (see above). Fully labeled ^{15}N-creatine was obtained from Cambridge Isotope Labs (Andover, MA). For samples at Stn 3, at the end of the incubation the dissolved NH_4^+ pool was isolated via solid phase extraction (Dudek et al. 1986, Brzezinski 1987) and its ^{15}N enrichment determined so that NH_4^+ uptake rates presented could be corrected for isotope dilution (Bronk et al. 2014). The limit of detection for creatine uptake was estimated by calculating the creatine uptake rate necessary to produce an increase in PN atom % enrichment twice that of our analytical precision, which was 0.00069 for the set of 10 isotope standards analyzed with the creatine uptake samples presented here. When this was done for each of the measured rates, limits of detection were estimated at 0.002 to 0.010 nmol l^{-1} h^{-1}.

Identification of creatinase and creatine kinase genes in MMETSP dataset

All transcriptome libraries from the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) (Keeling et al. 2014) were downloaded, representing 393 unique strains after discarding 17 low quality assemblies. Assemblies of combined data for each strain, as generated by the National Center for Genome Resources (NCGR), were used for downstream analysis. Protein predictions were generated for each assembly using Transdecoder (Haas et al. 2013) supplemented with Pfam hits and BLASTp against a filtered Eukaryote-only UniProt-Swissprot database to reduce hits to bacteria known to contaminate some assemblies. Identification of putative homologs was conducted using a baiting algorithm (bait_homologs), as previously described (Yang & Smith 2014). The protein sequences for creatine kinase (GenBank: EED89560.1) and creatinase (GenBank: EED87073.1) from *T. pseudonana* CCMP1335 were used as bait sequences against all predicted peptides from the MMETSP dataset using blastp (E = 10, 5 hits query^{-1}). Hits were initially aligned using either MAFFT (Katoh & Standley 2013) (using options 'genafpair' and 'maxiterate 1000') when clusters contained fewer than 1000 sequences or the iterative PASTA aligner (Mirarab et al. 2015) if sequence clusters were larger than 1000. Alignments were refined twice by removal of poorly aligned sequences as described above, which may indicate misassembly, clustering errors, and/or protein prediction errors. Phyutility was used to remove poorly aligned positions with more than 10% missing data for smaller (<1000 sequences) or 1% for larger (>1000 sequences) clusters of homologs. Phylogenetic trees were built of each sequence cluster using either RAxML (Stamatakis 2014) (PROTCATWAG substitution model) for smaller alignments (<1000 sequences) or FastTree2 (Price et al. 2010) (WAG substitution model) for larger clusters. Terminal branch tips longer than 10 times their sisters, with a relative length of >1.0 or an absolute length of >2.0, were subsequently removed. Paraphyletic and monophyletic tips representing isoforms were also removed, keeping only the sequence with fewest ambiguities. Internal branches longer than 1.0 were cut to separate deep paralogs, keeping the subtree containing the bait sequences. A second round of refinement used a relative length of 0.5 and an absolute length of 1.0, cutting internal branches longer than 1.0. The final alignment was generated using MAFFT (Katoh & Standley 2013) (using options 'genafpair' and 'maxiterate 1000'), and aligned with RAxML (Stamatakis 2014) to infer an initial maximum likelihood phylogenetic tree (PROTCATWAG substitution model). To verify similar domain structures between homologs, HMMER (http://hmmer.janelia.org) was used to find conserved PFAM domains among each homolog (E = 1e-5; 40% overlap with domains). Finally, only sequences that produced hits to all domains found in bait sequences were considered to be positive hits.

RESULTS

The diatom *Thalassiosira pseudonana* was analyzed in order to test if creatine can be detected as a cellular metabolite. This diatom was chosen because genomic analysis had previously suggested the potential for creatine bio-synthesis (Armbrust et al.

2004), but direct evidence of the presence of creatine in algae has not been presented in the literature. Using MS analysis, we were able to detect creatine in filtered cells of *T. pseudonana* (Fig. 1). While not quantitative, these data indicate that this diatom does appear to contain creatine as a cellular metabolite and may indeed be a potential source of creatine in the environment.

To gain better insight into how widespread the potential for creatine metabolism might be among the diverse array of eukaryotic microbes found in the oceans, an analysis of the MMETSP (Keeling et al. 2014) was conducted. The MMETSP represents the largest exome dataset for marine eukaryotic microbes to date, and was interrogated with respect to the presence of homologs for creatinase and creatine kinase sequences (Fig. 2) by using the gene sequences derived from the genome of *T. pseudonana* as a reference. This analysis indicated that out of 393 marine protist species available from MMETSP, 293 and 130 produced transcripts that potentially encode creatinase or creatine kinase, respectively (Fig. 2). Both functions appear phylogenetically widely distributed with some notable differences. For example, 19 out of 31 haptophyte transcriptomes contained a transcript for creatinase, but only one haptophyte appeared to produce transcripts for creatine kinase. Similarly, 9 out of 12 Amoebozoa produced creatinase transcripts, while only one amoeban transcriptome encoded creatine kinase. Almost all heterokonts (Stramenopiles), which includes the diatom lineage, produced a transcript for creatinase (130 out of 147), and about half (71 out of 147) expressed a gene homologous to creatine kinase (Fig. 2).

Creatine has the potential to serve as a C or N source to bacteria, and the potential for bacterial utilization of creatine was therefore investigated via bioinformatic analysis of existing sequence database information. To do so, the gene for creatinase from *Pseudomonas putida* KT2440, which has been functionally described (Schumann et al. 1993), was used as a reference sequence. A BLASTp search of this sequence against the National Center for Biotechnology Information (NCBI) NR database yielded 1742 blast hits. Creatinase is a member of a large di-peptidase protein family and BLASTp results were therefore further restricted to all hits with >50% identity and an E-score <1e-150, thereby excluding di-peptidases. This yielded 553 sequences, all of which are annotated as creatinases in GenBank. Taxonomic information was analyzed using MEGAN (Huson et al. 2007) at the genus level, which revealed actinobacterial, firmicute, and proteobacterial lineages (Fig. 3). The greatest number of taxa were observed in the *Proteobacteria*, accounting for 78% of observed genera. Among these, the alpha-*Proteobacteria* were the largest

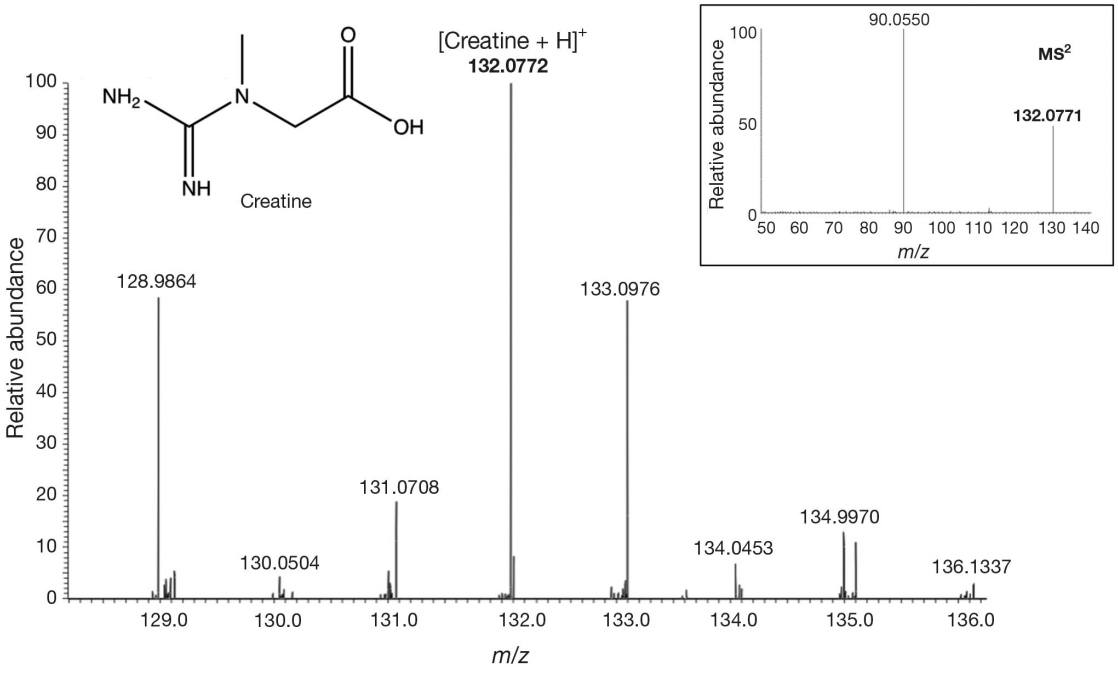

Fig. 1. Single-cell mass spectrometry (MS) analysis of creatine (skeletal formula shown in upper left) from *Thalassiosira pseudonana*. Creatine was detected as the protonated species with a mass to charge ratio (*m/z*) of 132.0772 at a high resolution (100 000), and the identification was confirmed by collision induced dissociation tandem MS (MS^2) analysis (inset)

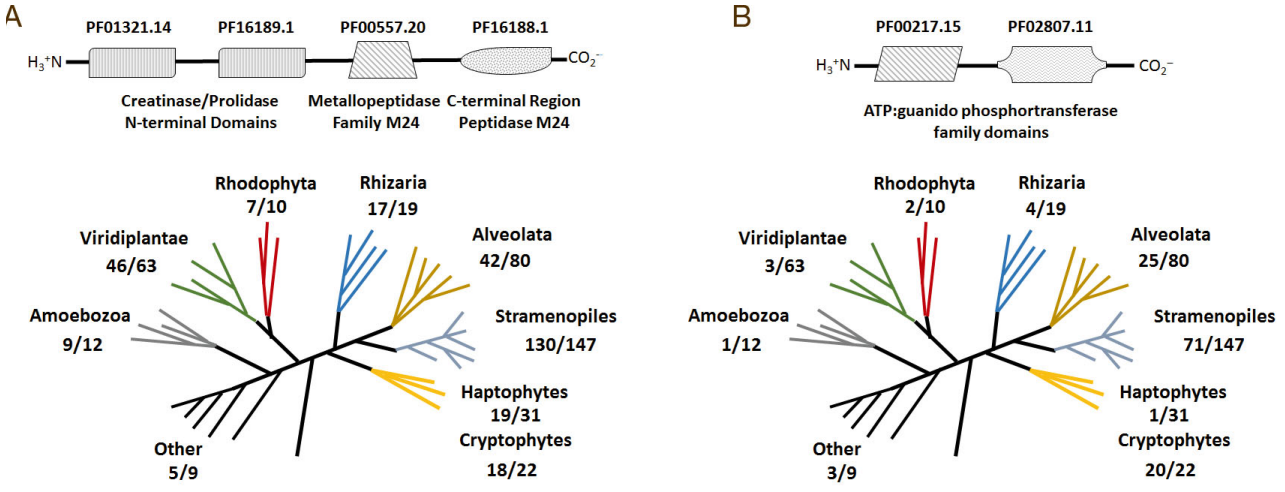

Fig. 2. Protein domain structure and phylogenetic distribution of homologs to (A) creatinase and (B) creatine kinase genes from *Thalassiosira pseudonana* in the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) dataset. Pfam identifiers are shown above symbolic representations of protein domains. Numbers at each of the branches of the dendrogram indicate the number of datasets that contain requisite homologs out of the whole MMETSP dataset

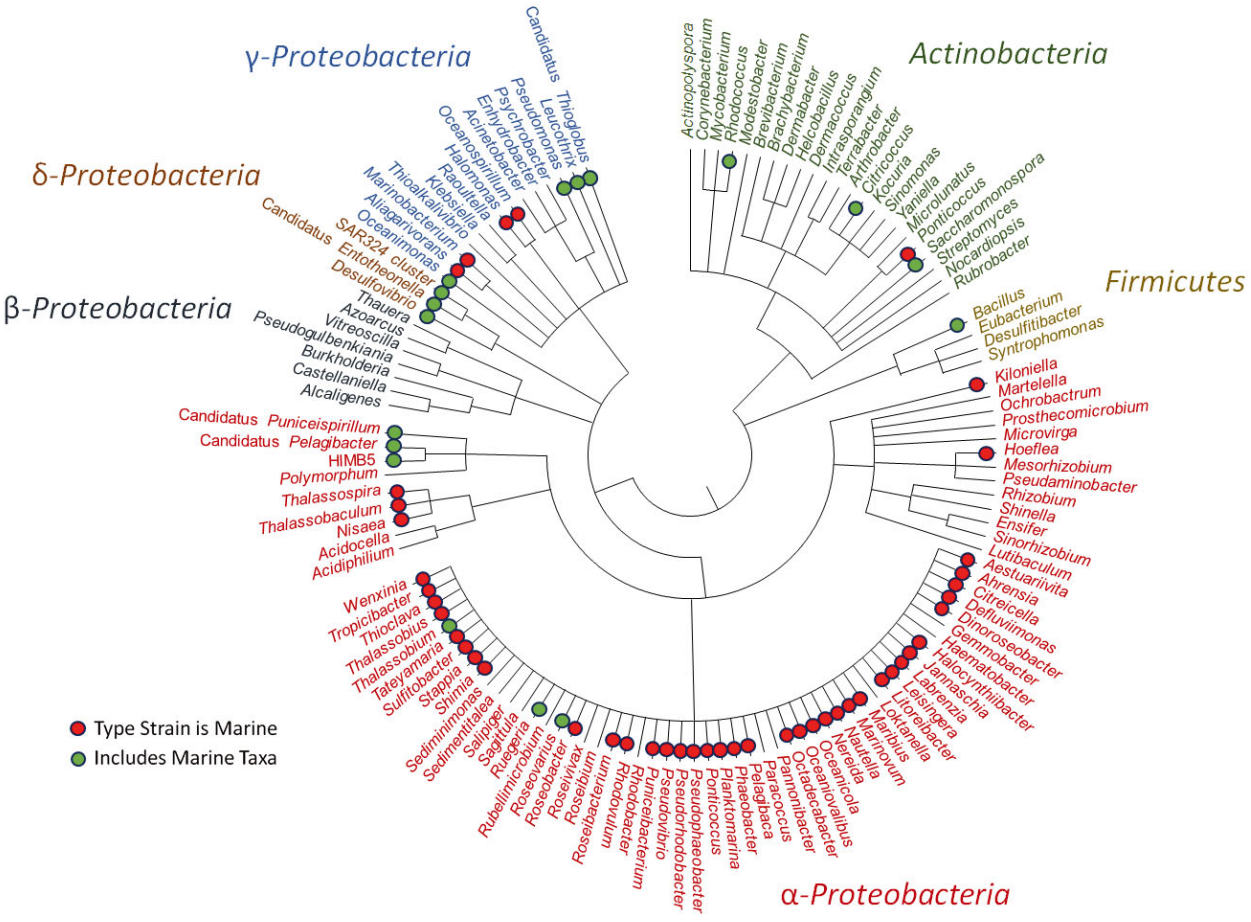

Fig. 3. Phylogenetic distribution of protein sequences in the NCBI NR database annotated as 'creatinase'. Genus level of classification is shown, and genera with type strains originating from marine environments are marked with red circles. Green circles indicate genera that contain well-known marine lineages, but for which type strains are either not marine or for which formal descriptions of the genus are not available. Genera without circles, to our knowledge, do not contain marine strains

Table 1. Test of growth on creatine as a C or N source by marine *Roseobacter* strains. +: growth observed after 2 wk; –: no growth

	Treatment			
	A	B	C	D
Substrate				
Glucose	10 mM	10 mM	–	–
Creatine	–	10 mM	10 mM	–
Strain				
Roseobacter denitrificans Och114	–	+	–	–
Roseobacter litoralis Och149	–	+	–	–

group, accounting for 59% of all taxa. Most striking, a large proportion of the genera associated with creatinase enzymes in GenBank either contain marine genera (>50%) or are known from their type strain, which is of marine origin (>37% of observed genera). However, we note that many of these annotations rely on database homology searches, and it is often not clear if ascribed functions truly reflect potential activities. The marine proteobacteria *Roseobacter litoralis* Och149 and *Roseobacter denitrificans* Och114 were among the strains that contained putative creatinase genes and in order to confirm their potential to utilize creatine, they were grown in minimal seawater medium containing 10 mM glucose, 10 mM creatine, or 10 mM of both (Table 1). After 2 wk of incubation, growth was only observed in the presence of both glucose and creatine. This indicates that creatine can be used as a N but not as a C source by these *Roseobacter* strains.

To further investigate the enzymatic properties of creatinases found in marine bacteria, the respective genes observed in the genomes of *R. litoralis* Och149 and *R. denitrificans* Och114 were therefore sub-cloned. Both C-terminal and N-terminal hexa-histidine tagged fusion constructs were generated for each of the genes and products were overexpressed in *E. coli* (Fig. 4). Purified protein was readily obtained for N-terminally His-tagged fusion proteins from the soluble protein fraction for both strains, but C-terminally tagged creatinase was only obtained from *R. denitrificans* Och114. Repeated attempts to purify C-terminally labeled creatinase from *R. lito-*

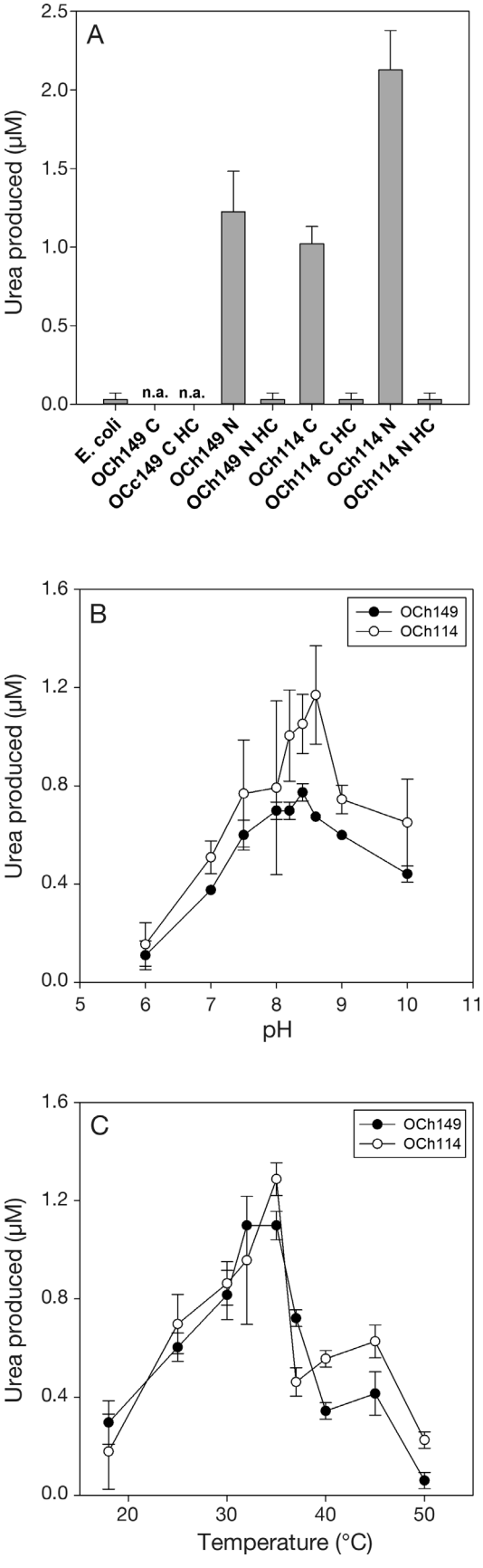

Fig. 4. Creatinase enzyme assay results. Requisite genes were cloned from *Roseobacter litoralis* Och149 and *Roseobacter denitrificans* Och114 and overexpressed in *E. coli*. Error bars indicate standard deviations calculated from triplicate measurements. All assays were performed with 5 mg of purified protein. Enzyme activity was measured by monitoring urea formation in the presence of creatine. (A) All gene products were cloned to include hexa-histidine tags at the C-terminal (labeled as C) or N-terminal (labeled as N); HC: heated controls. The first bar (*E. coli*) indicates activity in lysate of *E. coli* that does not contain an expression plasmid; 'n.a.': no activity. (B) Enzyme activity of purified creatinases across a pH range from 6 to 10 for both *Roseobacter* strains (Och149 and Och114); (C) enzyme activity observed for purified creatinase across a temperature range of 18 to 50°C

ralis Och149 yielded no protein product. Further, when activities were compared among C- and N-terminally labeled creatinase from *R. denitrificans* Och114, significantly greater activity was observed (*t*-test; $p < 0.005$) for the N-terminally labeled product (Fig. 4A). Subsequent analysis of enzyme characteristics (Fig. 4B,C, Table 2) was therefore limited to the N-terminally His-tagged creatinases. The highest activity of creatinases, measured as urea, was observed at a pH of 8.2 to 8.4, consistent with the pH of seawater (Fig. 4B). The temperature optimum of creatinases from both strains was ca. 35°C, and a rapid decline in activity was observed above this temperature (Fig. 4C). Protein sequence analysis does not indicate the presence of export signals of transmembrane helices, and purification efficiency was highest from soluble protein fractions, indicating that creatinase in *Roseobacter* sp. is likely a soluble cytosolic protein. The influence of NaCl, $MgSO_4$, $MgCl_2$, and Na_2SO_4 in assay buffers was tested (data not shown), and highest relative enzymatic activities were observed in 25 mM NaCl or 25 mM $MgSO_4$. The highest creatinase activities were observed in the absence (i.e. 0 mM) of $MgCl_2$ and Na_2SO_4. The determination of kinetic properties resulted in a K_m of 25 to 27 mM with a V_{max} of 3.3 to 4.4 µM mg^{-1}.

Given the potential for creatine utilization by marine bacteria (see above), its concentration and uptake rate were quantified in samples collected from the equatorial Pacific Ocean (Fig. 5, Table 3). Creatine was detectable in all samples and was greater at the surface than the DCM, ranging between 20 and 200 nmol N l^{-1}. Uptake rates ranged between 0.02 and 0.66 nmol N l^{-1} h^{-1}, with higher rates at the surface. Uptake rates for other N sources were not determined at the time, and we are unaware of published data for specific N utilization rates in the equatorial

Table 2. Kinetic properties of purified creatinase enzymes from 2 *Roseobacter* strains. Rate experiments performed at 33°C by running triplicate samples at 10 mM increments in a concentration range from 1 to 100 mM (total n = 30). V_{max} and K_m as determined via Lineweaver-Burke plot analysis

Creatinase	K_m (mM)	V_{max} (µM mg^{-1} min^{-1})
Roseobacter litoralis Och149	27.4 ± 6.1	4.4 ± 0.3
Roseobacter denitrificans Och114	25.5 ± 2.6	3.3 ± 0.1

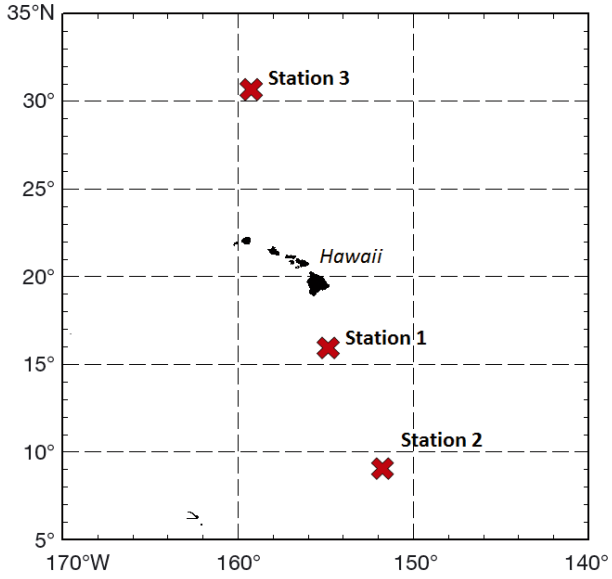

Fig. 5. Sampling locations in the equatorial Pacific south of Hawaii. Samples were collected on 2 separate cruises. Stns 1 and 2 were sampled in September and October 2014 aboard the R/V 'New Horizon' to conduct ambient creatine concentration and uptake rate measurements. Reference data for dissolved free amino acid and ammonium uptake were generated on a prior cruise (Stn 3) in May 1999 aboard the R/V 'Melville'

Table 3. Mean creatine concentrations and absolute uptake rates (n = 2) measured in the equatorial Pacific. Mean ± SD of dissolved free amino acids (DFAAs), ammonium (NH_4^+), and urea uptake rates were measured in the same region on a cruise of opportunity in May 1999, and are shown in *italics* to distinguish measurements obtained on different cruises

Station	Depth (m)	Creatine concentration (nmol N l^{-1})	Specific creatine uptake (h^{-1})	Absolute creatine uptake (nmol N l^{-1} h^{-1})	*Absolute DFAA uptake* (nmol N l^{-1} h^{-1})	*Absolute NH_4^+ uptake* (nmol N l^{-1} h^{-1})	*Absolute urea uptake* (nmol N l^{-1} h^{-1})
1	5	105 ± 32	0.0005 ± 0.0004	0.50 ± 0.39	–	–	–
	110	19 ± 14	0.00002 ± 0.00000	0.08 ± 0.02	–	–	–
2	5	171 ± 46	0.0019 ± 0.0010	0.66 ± 0.34	–	–	–
	140	79 ± 15	0.0004 ± 0.0001	0.02 ± 0.03	–	–	–
3	*10*	–	–	–	*1.82 ± 0.09*	*12.53 ± 9.52*	*3.11 ± 0.52*
	100	–	–	–	*1.03 ± 0.13*	*4.28 ± 2.01*	*1.25 ± 0.35*

Pacific. Therefore, we also present unpublished data from a cruise of opportunity showing the only amino acid uptake rates we are aware of for this region, as well as data for NH_4^+ and urea uptake measured on the same cruise (labeled as Stn 3 in Fig. 5 and Table 3) in order to compare creatine to other low molecular weight N substrates. Consistent with most studies, the highest rates measured were for NH_4^+. Uptake rates for all substrates were higher at the surface than at the deeper station. Comparing the 2 cruises, creatine utilization at Stns 1 and 2 were 27 to 36% of DFAA uptake at the surface and 2 to 8% of DFAA uptake at depth.

DISCUSSION

Bacteria, phytoplankton, zooplankton, and metazoans are all potential environmental sources of dissolved free amino acids (DFAAs). Bacteria are not known to be able to synthesize creatine, and data for zooplankton are not available. It is also unclear whether, and to what degree, metazoans are a potential a source of creatine in the oligotrophic ocean, possibly in the context of diurnally occurring vertical migration. Recent genetic evidence suggests that some algae are capable of producing creatine (Armbrust et al. 2004, Allen et al. 2006), and MS data presented here for the diatom *Thalassiosira pseudonana* supports this notion by demonstrating creatine as a cellular metabolite (Fig. 1). Analysis of transcriptome data from the MMETSP project indicates that creatine metabolism may be a widespread genetic trait among marine protists (Fig. 2), though some lineage-specific differences were observed. The role of the detected putative creatine kinase and creatinase genes in these organisms remains unknown. It is possible that creatinase mainly serves as a mechanism for seawater-derived creatine catabolism, thereby enabling cells to utilize this substrate as a nitrogen source under N-depleted conditions. Creatine kinase, however, typically serves to synthesize phosphocreatine, an energy storage molecule that allows rapid regeneration of the ATP pool. Beyond metazoan muscle tissue, phosphocreatine has been implicated in flagellar ATP generation in urchin and mammalian spermatozoa (Tombes et al. 1987, Huszar et al. 1997). In sea urchin sperm, specific inhibition of creatine kinase leads to flagellar paralysis (Quest & Shapiro 1991), suggesting a possible role of this enzyme in flagellar motility common to many protists. Centric diatoms, such as *T. pseudonana*, produce flagellated gametes, and it is possible that phosphocreatine serves as a means to power flagellar motility in these cells.

DON concentrations in seawater are generally highest near the surface and range between 3 and 5 µmol N l^{-1} in tropical waters, where they can contribute >90% of total dissolved N. Measurements of DFAAs range between 0.001 and 1.4 µmol N l^{-1}, typically representing ~1.2 to 12.5% of the total DON pool (Sipler & Bronk 2015). Given that creatine concentrations may be in the range of 0.019 to 0.171 µmol N l^{-1}, this suggests that creatine may account for a significant portion of the DFAA pool in oligotrophic waters, though several important caveats need to be noted. First, our creatine measurements exhibited relatively high variability. Second, we note that careful efforts to eliminate creatine contamination (e.g. from finger grease) are necessary during lab sample preparation and analysis. Creatine measurements may therefore be a potential over-estimation of actual ambient concentrations. However, uptake was observed at both stations and depths. Creatine uptake rates were low compared to NH_4^+ uptake, but they were significant with respect to rates observed for DFAA utilization (Table 3), though we note that our ability to compare datasets from these 2 cruises of opportunity is limited without additional reference data about community composition and activity.

Phytoplankton are a known source of DFAAs via cellular exudates. In the diatom *Chaetoceros affinis*, for example, amino acid exudation is highest during exponential growth and includes a range of polar and non-polar amino acids such as aspartic acid, glutamic acid, serine, glutamine, glycine, alanine, valine, and leucine (Myklestad et al. 1989). Amino acids with N-containing side chains, such as arginine and asparagine, were also observed, though these were released during the stationary phase (Myklestad et al. 1989). Similarly, in the diatom *Skeletonema costatum*, 4% of photosynthetic production is released during exponential growth and the stationary phase (Granum et al. 2002). Products released from healthy cells were composed of ca. 5% free amino acids (measured as C), while the composition of the released amino acids depends on growth phase and differed from intracellular amino acid pools (Granum et al. 2002). Others have noted that phytoplankton-produced DFAAs also vary in amount and composition among species (Sarmento et al. 2013). Measurements of creatine were not reported in these studies, but the observation that DFAAs are readily released by phytoplankton in culture supports the notion that phytoplankton are a potential creatine source in natural systems.

While DON release has important implications for phytoplankton physiology, the primary consumers of creatine in the environment are likely bacteria, though we recognize that a broad array of phytoplankton are capable of using a wide variety of low molecular weight organic compounds (Bronk & Flynn 2006). It has been noted that creatinases can be found in strains of *Alcaligenes*, *Actinobacillus*, *Arthrobacter*, *Flavobacterium*, *Bacillus*, *Paracoccus*, *Pelagibacter*, and *Pseudomonas* (Zhi et al. 2009). The same paper also indicated that *Arthrobacter nicotianae* strain 02181 appears to be unusual in being able to utilize creatine as both a C and N source, albeit inefficiently, and is consistent with observations described here for marine *Roseobacter*. The phylogenetic breadth of creatine utilizers is also much broader than previously noted and appears to include at least 125 genera, over a third of which have type strains of known marine origin (Fig. 3). Included in this list are some of the ocean's most dominant bacterial taxa, including *Pelagibacter ubique*, the SAR324 cluster, *Marinobacterium*, *Halomonas*, *Oceanospirillum*, *Marinobacter*, and the aforementioned *Roseobacter*.

We note that bioinformatic data mining has its limitations, since functional annotations are often based solely on sequence homology. We therefore set out to characterize the genes annotated as creatinase in the genomes of *R. litoralis* Och149 and *R. denitrificans* Och114, which were the first described members of the *Roseobacter* group (Buchan et al. 2005). Both are pink-pigmented bacteria that contain bacteriochlorophyll *a* and were isolated from marine algal strains (Shiba 1991). The family *Rhodobacteraceae* now includes at least 17 genera, which can be found in virtually every marine habitat (Wagner-Döbler & Biebl 2006). Of these, 11 are associated with creatinase genes in GenBank (Fig. 3), including the genera *Oceanicola*, *Loktanella*, *Sagitula*, *Octadecabacter*, *Ruegera*, *Sulfitobacter*, *Roseobacter*, *Roseovarius*, *Roseovivax*, *Salipiger*, and *Jannaschia* sp.. *Roseobacter* abundances vary among environments, but can be as much as 25% of the bacterial populations in coastal and polar environments (Wagner-Döbler & Biebl 2006), underscoring their environmental significance. Many strains are known for the ability to perform aerobic anoxygenic photosynthesis (AAnP) and *Roseobacter* appear to be critical to the ocean's sulfur cycle due to their ability to degrade the algal osmolyte dimethylsulfoniopropionate (DMSP).

Roseobacter strains also are known for symbiotic interactions with organisms that are known to or potentially can produce creatine. It is therefore not surprising that the genomes of many marine *Roseobacter* sp. contain the functional potential for creatine utilization, thereby permitting inter-species N transfer in requisite interactions. For example, *Roseobacter* live in close interaction with mollusks, where they potentially degrade or modify dinoflagellate toxins (Smith et al. 2001), and the production of algal lytic compounds by *Roseobacter* can impact the dynamics of algal blooms (Amaro et al. 2005). *Roseobacter* often account for a large proportion of bacteria associated with planktonic algae, particularly dinoflagellates (González et al. 2000), and it was shown that as much as 50% of bacteria associated with *Pfiesteria* may belong to the *Roseobacter* lineage (Alavi et al. 2001). Interactions with metazoans have also been described, but are typically best known from pathogenic relationships such as juvenile oyster disease, or white plaque and black band coral disease (Boettcher et al. 2000, Cooney et al. 2002, Pantos et al. 2003).

Data for enzymatic properties of *Roseobacter* creatinases reported here are consistent with functional cytoplasmic enzymes. Creatinase in *Pseudomonas putida* has been described as a 2 domain peptide that forms a 91 kDa homo-dimer that does not contain disulfide bonds (Schumann et al. 1993). The enzyme is not known to contain co-factors and several metal ions, including Cu^{2+}, Fe^{3+}, Hg^{2+}, and Zn^{2+} are known to inhibit activity (Zhi et al. 2009). The pH optima for previously characterized creatinases have ranged from 6.7 (*P. putida*) to 8 (*Alcaligenes* sp.) (Roche & Lacombe 1950, Matsuda et al. 1986), and *Roseobacter* creatinases therefore have the highest pH optimum reported to date at ca. 8.2 to 8.4. This pH is more consistent with the pH of seawater than a likely cytosolic pH of *Roseobacter* cells, and we therefore investigated the potential for protein export. Export would allow extracellular hydrolysis and release of urea that could then serve as an external N source via urea uptake and hydrolysis. Creatinases from *R. litoralis* Och149 and *R. denitrificans* Och114, however, do not appear to contain common transport signal peptides, cleavage sites, or twin arginine signals. This is consistent with experiments on the influence of the major seawater ions (data not shown), which indicated a drop in enzymatic activity above 25 mM NaCl or $MgSO_4$. Previously reported specific activities ranged at the extreme from 0.0014 µmol min^{-1} mg^{-1} for crude enzyme extracts (Dai et al. 2015) to 124.4 µmol min^{-1} mg^{-1} for a high activity purified enzyme from *A. nicotianae* (Zhi et al. 2009). More typical values appear to be in the middle of this range, consistent with observations reported here (Table 2). For example, activities of 8.5 µmol min^{-1} mg^{-1} and 6.1 µmol min^{-1} mg^{-1}

have been reported for purified enzymes from *Alcaligenes* sp. and *Paracoccus* sp., respectively (Matsuda et al. 1986, Wang et al. 2006). These specific activities, absence of transport signals, and relatively high K_m values for *Roseobacter* creatinases all lead to the conclusion that these enzymes likely serve to liberate N intracellularly and are perhaps more significant in environments where creatine is in relatively high abundance, such as symbiotic interactions with creatine-producing algae and metazoans.

In summary, data presented here are the first measurements of ambient creatine concentrations in seawater and demonstrate that creatine is utilized at a potentially significant rate when compared to the rate of DFAA turnover. We further describe the first data for kinetic properties of purified creatinases from marine bacterial strains, identified creatine as a metabolite in *T. pseudonana*, and explore the phylogenetic breadth of creatine metabolism in both bacteria and marine protists. Overall, these data point to widespread potential of creatine metabolism in marine microbial communities, though a number of questions remain. First, at the cellular level, the physiological role of creatine metabolism in protists remains unclear. Second, spatial and temporal variability may have a significant impact on the presence of creatine and turnover rates in the oceans and remains to be explored in the context of larger oceanographic features and processes.

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