Adaptive Radiation of Marine Ammonia-oxidizing Archaea

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Abstract

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We report here on the basic physiology of two new isolates of marine ammonia-oxidizing archaea (AOA) and their relationship to *Nitrosopumilus maritimus*, the first isolated representative of the marine AOA. Although all isolates are closely related members of the Group 1 clade, they exhibit strikingly different growth responses to differences in pH, salinity, temperature, and light. Notably, strain PS0 is capable of sustaining nearly 80% of ammonia oxidation activity at a pH as low as 5.9, challenging previous generalizations of marine AOA sensitivity to increases in ocean acidity. All strains showed only minor photoinhibition at 15 μE m^{-2} s^{-1}, demonstrating the plausibility of an AOA origin of the primary nitrite maximum in the euphotic zone. Comparative analyses also provided direct physiological confirmation of a capacity to utilize fixed carbon and urea as carbon and energy sources, respectively. Together, these findings highlight a remarkable adaptive capacity of marine AOA and new understanding of the physiological basis for the remarkable ecological success reflected by their high oceanic abundance, and possible future success in a changing ocean.

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Significance Statement

The ammonia-oxidizing archaea (AOA) have major influence on the form and availability of nitrogen in the marine environment. They are a major contributor to oceanic N2O release to the atmosphere and are a plausible indirect source of methane in the upper ocean. Thus, their sensitivity to ocean acidification and other physicochemical changes associated with climate change has global significance. We here report on the physiological response of marine AOA recently brought into pure culture. Although natural AOA populations have been reported to be highly sensitive to slight reductions in ocean pH, we now show that some marine AOA can retain most of their ammonia oxidation activity in a highly acidic ocean.

Introduction

The discovery of ammonia-oxidizing archaea (AOA), sometimes constituting up to nearly 40% of marine microbial plankton, challenged the traditional view of microbial controls of nitrogen speciation in the ocean (Sintes et al.; Karner et al., 2001; Kirchman et al., 2007; Agogue et al., 2008; Prosser and Nicol, 2008). Initial understanding of the physiological basis for high AOA abundance in the marine water column came from the demonstration that *Nitrosopumilus maritimus* SCM1 is an extreme oligotroph, having one of the highest affinities (low K_s) for ammonia yet observed in pure culture (Martens-Habbena et al., 2009). However, additional conclusions about physiological attributes that determine activity patterns in the marine environment were based primarily on indirect observations. Most notably, a capacity to use fixed carbon and urea as alternative carbon and energy sources, respectively, has been inferred from tracer, metagenomic, and metatranscriptomic studies (Ouverney and Fuhrman, 2001; Herndl et al., 2005; Ingalls et al., 2006; Teira et al., 2006; Hansman et al., 2009; Konstantinidis

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et al., 2009; Hollibaugh et al., 2011; Yakimov et al., 2011; Alonso-Saez et al., 2012; Tully et al., 2012). The contribution of AOA to endogenously generated nitrate in marine surface waters, of fundamental relevance to the source of nitrogen sustaining primary production in the photic zone, was suggested by relating nitrification activity and archaeal *amoA* gene (coding for the α-subunit of the putative ammonia monooxygenase) distribution patterns (Horak et al.; Caffrey et al., 2007; Mincer et al., 2007; Kalanetra et al., 2009; Church et al., 2010; Newell et al., 2011). A reported sensitivity of marine populations to minor reductions in pH, important to understanding the possible impact of ongoing ocean acidification on the marine nitrogen cycle, was inferred from a pH perturbation study (Beman et al., 2011). Although these reports addressed a number of questions central to understanding microbial controls of the marine nitrogen cycle, they lacked direct physiological confirmation. Thus, a more detailed understanding of the adaptive capacity of different AOA lineages is needed to more fully appreciate the potential impact of climate change on nitrogen cycling.

Although the enrichment of novel AOA strains belonging to Groups I.1a and I.1b from a variety of marine and terrestrial environments have been reported (de la Torre et al., 2008; Hatzenpichler et al., 2008; Jung et al., 2011; Lehtovirta-Morley et al., 2011; Santoro and Casciotti, 2011; Tourna et al., 2011; French et al., 2012; Kim et al., 2012; Mosier et al., 2012), no additional marine isolates have been reported since the isolation of *Nitrosopumilus maritimus* strain SCM1 (Konneke et al., 2005). We now report the isolation of two novel marine AOA strains from the Puget Sound estuary system in Washington State, significantly expanding the diversity of marine *Thaumarchaeota* available in pure culture. Despite relatively close phylogenetic relationships, the three marine isolates comprise physiologically distinct ecotypes of AOA, varying in their

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capacity to use different carbon and energy sources, and in their tolerance to differences in pH, salinity, and light. These distinctive differences are directly relevant to their possible contribution to nitrification in different marine environments – including lower salinity coastal regions, the photic zone of the upper water column, and in ocean waters of increasing acidity associated with a changing ocean.

Results

Enrichment and isolation of marine AOA. Enrichment cultures were initiated from Puget Sound main basin surface water (47.55 N, 122.28 W) and 50 m water from the Puget Sound Regional Synthesis Model (PRISM) Station P10 (47.91 N, 122.62 W) in Hood Canal, respectively. Our previous molecular surveys showed these two coastal waters to be dominated by AOA, rather than their ammonia-oxidizing bacterial counterparts (AOB) (Horak et al.; Huguet et al., 2010; Urakawa et al., 2010). Predominance of AOA was consistent with generally sub-micromolar concentrations of ammonia at these stations, concentrations too low to sustain cultured AOB, but sufficient to support AOA such as *N. maritimus* strain SCM1 (Martens-Habbena et al., 2009). Enrichment conditions were designed to simulate the low substrate availability and growth conditions of these marine systems, using filtered seawater from the two stations (0.22μm STERIVEX- GP filter unit, Millipore) supplemented with 2 μM NH_4Cl and incubated at 15 °C.

Once the enrichment cultures were stable to repeated transfer, they were transferred to artificial seawater media supplemented with 2 μM NH_4Cl and 100 μM α-ketoglutaric acid. Highly enriched AOA cultures were obtained following approximately 2 years of consecutive transfer of

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10% (vol/vol) late exponential phase subcultures into the same media. Comparable growth kinetics were observed at 50 µM and 2 µM ammonia. Thus, the higher concentration of ammonium was subsequently used for isolating the two new AOA strains from the enrichment cultures. Pure cultures were ultimately obtained by filtration of the enrichment cultures through a 0.22 µm Millex-GP syringe filter, and diluting the filtrate to extinction (see *SI Materials and Methods* for additional details). The two new oligotrophic AOA strains were designated HCA1 (Hood Canal station P10) and PS0 (Puget Sound main basin).

Exponential growth in synthetic seawater medium containing 500 µM ammonium and 100 µM α-ketoglutaric acid of both isolates was supported by the near stoichiometric oxidation of ammonia to nitrite (Fig. 1*A*, *B*). No growth was observed in medium containing α-ketoglutaric acid and no ammonium, supplemented with nitrite or nitrate as possible nitrogen sources. The maximum specific growth rates for strain HCA1 and PS0 were 0.55 d^{-1} and 0.23 d^{-1}, respectively. The growth rate of HCA1 was comparable to *N. maritimus* (0.65 d^{-1}) and the one described thermophilic AOA, "*Candidatus* Nitrosocaldus yellowstonii" (0.8 d^{-1}) (Konneke et al., 2005; de la Torre et al., 2008). The lower growth rate of PS0 was similar to the previously described pelagic AOA enrichments, CN25 and CN75 (0.17 d^{-1}) and to the estimated *in situ* growth rates of AOA in winter polar waters (0.21 d^{-1}) (Santoro and Casciotti, 2011; Alonso-Saez et al., 2012). Since both strains were of similar shape and size to SCM1 (Fig. 1*C*, *D*; see description below), calculation of maximum ammonia oxidation activities were based on the reported cellular biomass of SCM1, using a factor of 10.2 fg protein $cell^{-1}$ (Martens-Habbena et al., 2009). The values for HCA1 (24.5 µmol NH_4^+ mg $protein^{-1}$ h^{-1}) and PS0 (12.2 µmol NH_4^+ mg $protein^{-1}$ h^{-1}) (Table S1) were both lower than SCM1 (51.9 µmol NH_4^+ mg $protein^{-1}$ h^{-1}) and characterized

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AOB (30-80 μmol NH_4^+ mg protein^{-1} h^{-1}) (Ward, 1987; Prosser, 1989). These values were within the range estimated for *in situ* cell-specific rates of a natural marine community (0.2-15 fmol NH_4^+ cell^{-1} d^{-1}) (Santoro et al., 2010).

Morphology and phylogeny. The morphologies of strains HCA1 and PS0, as characterized by transmission electron microscopy (Fig. 1*C*, *D*), are very similar to *N. maritimus* (Konneke et al., 2005). Both are small rods, with a diameter of 0.15 to 0.26 μm and a length of 0.65 to 1.59 μm. Unlike "*Ca.* Nitrosoarchaeum limnia" (Mosier et al., 2012), no flagella were observed by electron microscopy. Cells reproducing by typical binary fission were also observed in TEM images of mid-exponential phase cultures.

Close phylogenetic relationships among HCA1, PS0, and *N. maritimus* strain SCM1 were established by 16S rRNA and *amoA* gene sequencing. All were affiliated with *Thaumarchaeota* Group I.1a, together forming a monophyletic clade sharing >95% *amoA* and >99% 16S rRNA gene sequence identity (Fig. 2, S1). In contrast, "*Candidatus* Nitrosoarchaeum limnia", previously described in an enrichment from a low-salinity estuarine system in California, differed from the new isolates by greater than 4% and 7% sequence divergence of the 16S rRNA and *amoA* genes, respectively (Blainey et al., 2011). The more divergent symbiotic archaeon "*Candidatus* Cenarchaeum symbiosum" shares less than 95% and 84% sequence identity with the 16S rRNA and *amoA* genes of novel strains (Hallam et al., 2006). All share less than 90% and 80% identity of the 16S rRNA and *amoA* genes, respectively, of the cultured soil representatives "*Candidatus* Nitrosotalea devanaterra" and *Nitrososphaera viennensis* (Lehtovirta-Morley et al., 2011; Tourna et al., 2011).

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Relationships between growth rate, temperature, salinity, and pH. The temperature dependent growth kinetics of the new isolates differed significantly from strain SCM1. The highest growth rates for strains HCA1 and PS0 were observed at 25 °C and 26 °C, respectively, in contrast to a maximum at 32 °C for strain SCM1 (Fig. S2*A*-*C*). No growth (nitrite production) could be detected for SCM1 at 10 °C, whereas the two new isolates continued to grow at 10 °C. An Arrhenius analysis revealed a good linear relationship between the natural logarithm of the ammonia oxidation rate and the inverse of the absolute temperature (Fig. S2*D*). The inferred activation energy (78.25 kJ mol^{-1}) and Q_{10} value (2.89) of SCM1 were somewhat greater than strain HCA1 (E_a =67.67 kJ mol^{-1}, Q_{10}=2.62) and strain PS0 (E_a =64.20 kJ mol^{-1}, Q_{10}=2.49). These values are comparable to those estimated for estuarine nitrifying bacteria representatives (E_a =67.4-82.5 kJ mol^{-1}, Q_{10}=2.7-3.3) (Helder and Devries, 1983) (Table S2).

Growth of strains SCM1 and HCA1 was restricted to pH values between 6.8 and 8.1, with the highest ammonia oxidation rates observed at pH 7.3 (Fig. 3*A*). In contrast, strain PS0 grew well at significantly lower pH values, having a maximum growth rate at pH 6.8 and maintaining nearly 80% of its maximum growth rate at pH values as low as 5.9. No growth of either SCM1 or HCA1 was observed at this pH. However, at pH values closer to that of open ocean surface waters (~ 8.1), the growth of PS0 was depressed relative to the other two isolates. Thus, strain PS0 appears well adapted to the more acidic waters of the Puget Sound main basin from where it was isolated, having a pH range of 7.71 to 8.05 near the surface and of 7.70 to 7.83 at depths greater than 100m (Feely et al., 2010). No ammonia oxidation was observed at pH 8.7 for any of

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the strains, which is consistent with observations of other neutrophilic AOA (Jung et al., 2011; Tourna et al., 2011; Kim et al., 2012).

Salinity is also a significant environmental variable, particularly in coastal regions influenced by varying inputs of terrestrial freshwater. The Hood Canal fjord is influenced by seasonally varying riverine and surface runoff sources of freshwater, and by the intrusion of low pH and high salinity water from seasonal coastal upwelling of deep ocean water (Moore et al., 2010). All strains grew best at mid-salinity (25 to 32‰), but differed markedly in response to low and high salinity conditions (Fig. 3*B*). Strain SCM1 grew well at high salinities (35 to 40‰), was significantly inhibited at 20‰ salinity, and ceased to grow at 15‰ salinity. Strains HCA1 and PS0 were less inhibited at 20‰ salinity and still remained active at 15‰ salinity. However, unlike SCM1, both of these strains were significantly inhibited at 40‰ salinity. Since 40‰ salinity is much higher than the normal oceanic environment, these differences likely reflect strain specific adaptations to the varying salinities intrinsic to this coastal system.

Photoinhibition. The influence of light on growth kinetics was examined by controlled exposure of the isolates to polychromatic light from a cool, white fluorescent lamp. Growth rate was measured at different intensities of illumination and under three different illumination regimes: continuous dark, continuous illumination, and a 14h dark/10h light cycle (to mimic a natural diurnal cycle). Differential growth during light and dark periods of the imposed diurnal cycle was also examined. All isolates were differentially inhibited. Strain SCM1 was significantly less photosensitive (Fig. 4*A*) than the other two isolates when exposed to a diurnal light cycle, showing no apparent inhibition (relative to cultures incubated in the dark) at low light fluxes (15

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and 40 μE m^{-2} s^{-1}) and retaining about 20% of its maximum growth rate at the highest light intensity examined (180 μE m^{-2} s^{-1}). However, under continuous illumination at 120 μE m^{-2} s^{-1} this strain was completely inhibited (Fig. 4*B*). Although exhibiting somewhat greater light sensitivity, the response of strain HCA1 was similar to SCM1, showing reduced specific growth rates of 11% and 22% at 15 and 40 μE m^{-2} s^{-1}, respectively, and complete inhibition at the highest light intensity examined (Fig. 4*A*). Strain PS0 was the most light sensitive of the three AOA isolates, being partially inhibited at low light fluxes (19% and 39% inhibition at 15 and 40 μE m^{-2} s^{-1}, respectively), 80% inhibited at 60 μE m^{-2} s^{-1}, and completely inhibited at 80 μE m^{-2} s^{-1}.

The greater inhibition observed with continuous illumination relative to an imposed dark/light cycle suggested that partial recovery of activity was possible during the dark period. Dark recovery of strain HCA1 was shown by an increased growth rate during the dark period (Fig. 4*C*). To further evaluate dark recovery of completely light-inhibited cultures, strain SCM1 cultures were first incubated under continuous illumination at 60 μE m^{-2} s^{-1} until growth ceased (~ 100 hours) and then transferred to the dark. These cultures did recover, completely converting all ammonia to nitrite, but at a significantly reduced growth rate compared to dark-incubated controls (Fig. 4*D*). Thus, although the data confirmed sensitivity of AOA to light, they also suggest that potentially significant ammonia oxidation is possible in the upper water column through a normal diurnal cycle, and that only minor inhibition would be expected at ~15 μE m^{-2} s^{-1} under continuous illumination. This corresponds to light reaching the bottom of the photic zone in Hood Canal, near the nitrite maximum.

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Assimilation of organic carbon. Isolation of HCA1 and PS0 required the addition of a low concentration of α-ketoglutaric acid to the inorganic basal medium. Both strains oxidized approximately half of the added ammonia to nitrite following initial transfer from α-ketoglutaric acid supplemented into organic carbon-free media (1% inoculum), and failed to grow following a second transfer into organic carbon-free medium (Fig. 5*A*, *B*). The oxidation of half of the ammonia following the first transfer likely reflected either use of endogenous cellular reserves or the carryover of a low amount of α-ketoglutaric acid in the inoculum. As previously reported, strain SCM1 was capable of chemolithoautotrophic growth, oxidizing all added ammonia in the absence of an organic carbon supplement. However, SCM1 growth rate and cell yield were greater in cultures supplemented with α-ketoglutaric acid (Fig. 5*C* and Table S3). Since cell yield per mole of ammonia oxidized was greater for all strains in organic carbon supplemented media (from 93.4 to 112.6×10^{12} cells mol^{-1} NH_4^+ for SCM1, no growth to 80.8×10^{12} cells mol^{-1} NH_4^+ for HCA1, and no growth to 70.4×10^{12} cells mol^{-1} NH_4^+ for PS0) (Fig. S3), these results suggested that mixotrophic growth may be the preferred lifestyle of marine AOA.

Growth of strain PS0 on urea. The genetic potential for using urea to fuel nitrification has been reported for the marine group I *Thaumarchaeota* (Konstantinidis et al., 2009; Alonso-Saez et al., 2012). However, direct physiological support has to date been lacking. We therefore examined the capacity of each strain to grow in an ammonia free medium supplemented with approximately 100 μM urea. As predicted from the SCM1 genome sequence, previously reported to lack genes annotated for urea transport and degradation, no growth or nitrite production was observed for SCM1 cultures following more than 3 months of incubation. Likewise, no growth of strain HCA1 was observed in the urea medium. In contrast, strain PS0

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grew by near stoichiometric conversion of urea (~90 μM) to nitrite (~180 μM) in 17 days (Fig. S4) at a specific growth rate (0.18 d^{-1}) comparable to its growth on ammonia (0.23 d^{-1}).

Discussion

Through the expansion of the pure culture collection of marine AOA we can gain further understanding of the physiology and ecological significance of this globally significant assemblage of archaea. Since the successful isolation of the first ammonia-oxidizing archaeon (*Nitrosopumilus maritimus* strain SCM1) from a seawater aquarium (Konneke et al., 2005) using low ammonia concentrations for selective enrichment, the same general approach has been widely used to enrich additional AOA from soil, fresh water, marine and geothermal habitats (de la Torre et al., 2008; Hatzenpichler et al., 2008; Jung et al., 2011; Lehtovirta-Morley et al., 2011; Santoro and Casciotti, 2011; Tourna et al., 2011; French et al., 2012; Kim et al., 2012; Mosier et al., 2012). However, despite significant efforts to obtain new isolates from enrichment cultures, other than *N. maritimus* only *Nitrososphaera viennensis* strain EN76 from garden soil has been reported in pure culture (Tourna et al., 2011). In contrast to chemolithoautorophic growth of SCM1, *N. viennensis* and the new marine isolates require organic carbon, a requirement that may account for the virtual absence of publications describing additional isolates. Presumably the contaminating bacteria present in all described enrichments provide essential nutrients, and without appropriate nutrient supplementation the AOA cannot be maintained (Tourna et al., 2011; French et al., 2012; Kim et al., 2012). For example, the isolation of the soil AOA *N. viennensis* from an enrichment culture containing bacteria required the addition of an organic acid (pyruvate), presumably decoupling co-dependence (Tourna et al., 2011). However, pyruvate addition has not been sufficient for the isolation of AOA from other enrichment cultures (French

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et al., 2012), suggesting that different genotypes require different nutrient supplementation. It is possible that some adaptive changes associated with extended laboratory culture, here several years for each described strain, may have facilitated ultimate isolation. We also suspect that some patience is a requirement, since the successful isolation of the new slow-growing isolates described in this report was a multiple-year effort.

The three strains (SCM1, HCA1 and PS0) described here are relatively closely related members of a marine AOA lineage that differ markedly in basic physiological features. Their very different adaptive traits highlight the importance of culture-based analyses for informing environmental studies. Although strain SCM1 was isolated from a tropical marine aquarium, it shares high genomic similarity with marine metagenomic sequences and has an apparent half-saturation constant (K_s) for ammonia oxidation (133 nM) comparable to K_s values determined directly in open ocean waters (84-112 nM) (Horak et al.; Konneke et al., 2005; Martens-Habbena et al., 2009). The additional physiological characterization of this strain presented here, showing an optimum growth temperature near 30-33 $^{\circ}$C, and a preference for salinities (32-40 ‰) higher than the newly described coastal isolates, also suggests that *N. maritimus* strain SCM1 was originally native to tropical open ocean waters.

All isolates vary significantly in sensitivity to light and pH. These features relate directly to abiotic factors controlling their environmental distribution and response to increasing ocean acidity. The sensitivity of marine AOA to ocean acidification has been inferred from a limited number of short-term studies of experimentally acidified ocean water (Huesemann et al., 2002; Beman et al., 2011; Kitidis et al., 2011). For example, a recent study by Beman et al. (2011)

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reported significant inhibition of ammonia oxidation (8-38% reduction in rates) following relatively minor pH reductions of open ocean waters in which AOA were the dominant ammonia-oxidizing population (Beman et al., 2011). This effect was associated with the reported requirement for the use of ammonia (NH_3), not ammonium (NH_4^+), as a substrate for ammonia-oxidizing bacteria such as the marine *Nitrosococcus oceani* (Ward, 1987). The concentration of this form is significantly reduced by small reductions in pH. However, as yet there is no evidence that AOA have the same substrate requirement as AOB. In fact, the description of "*Candidatus* Nitrosotalea devanaterra", an acidophilic AOA growing optimally at pH values near 4, suggests that the NH_4^+ ion is more likely the substrate for this organism (Lehtovirta-Morley et al., 2011). Our observation that marine AOA strain PS0 grows well at pH 5.9 points to the existence of marine populations already well adapted to low pH and future changes in ocean acidity. If the ammonium ion is the preferred substrate, acidification may actually promote the growth of AOA, influencing oceanic production of N_2O now associated primarily with their activities (Santoro et al., 2011; Loscher et al., 2012).

Light is also implicated in controlling the activity and distribution of both ammonia oxidizers and nitrite oxidizers in the water column. The inhibitory effect of light on cultured AOB has been known for decades and attributed to photooxidative damage of the copper-containing ammonia monooxygenase (Hooper and Terry, 1973, 1974; Hyman and Arp, 1992). Archaeal photosensitivity was recently reported for marine, soil and fresh water AOA strains, including *N. maritimus* (French et al., 2012; Merbt et al., 2012). At a light intensity similar to the base of the euphotic zone (15 $\mu E\ m^{-2}\ s^{-1}$), Merbt et al. (2012) reported the growth of *N. maritimus* was almost completely inhibited with no evidence of dark recovery during an imposed diurnal cycle

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(Merbt et al., 2012). In contrast, we found only a marginal inhibitory effect of a comparable diurnal light regime on strain SCM1 (2.5% at 15 μE m^{-2} s^{-1}) and a similarly low inhibition of novel strains HCA1 and PS0 to this light regime. Even under continuous illumination, the growth rate of SCM1 was reduced by only 11.8% at this light flux. At higher intensities (40 and 60 μE m^{-2} s^{-1}) these isolates were capable of rapid dark recovery from photoinhibition, suggesting that ammonia oxidation in upper regions of the euphotic zone could follow a diurnal cycle. Although we have no explanation for the disparity between this and the previous study, these data provide physiological explanation for the high abundance and activity of AOA near the bottom of the euphotic zone as inferred by relating *amoA* gene and transcript abundance to *in situ* ammonia oxidation rate measurements (Horak et al.; Mincer et al., 2007; Beman et al., 2008; Santoro et al., 2010).

The requirement of the new isolates for organic carbon provided direct physiological support for previous isotopic studies suggesting both organic and inorganic carbon sources were assimilated by marine *Thaumarchaeota* (Herndl et al., 2005; Ingalls et al., 2006; Agogue et al., 2008; Hansman et al., 2009). There are obvious energetic advantages to the use of organic carbon to supplement carbon fixation. In addition, organic material might serve as an alternative source of reductant, other than electrons derived solely from ammonia, for the ammonia monooxygenase. Our demonstration that isolate PS0 is capable of using urea as an alternative energy source also provided the first direct physiological confirmation of an activity implicated by marine genomic data sets (Konstantinidis et al., 2009; Yakimov et al., 2011; Alonso-Saez et al., 2012). If the AOA have an affinity for urea comparable to their remarkably low K_s for ammonia, they may

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also effectively compete with phytoplankton for this reduced form of nitrogen, a question that can now be addressed using this new isolate.

The new physiological data now begin to constrain environmental variables influencing the distribution and activity of these remarkably successful organisms, now thought to play a fundamental role in the shaping of the marine nitrogen cycle. For example, the requirement of the new isolates for organic carbon suggests that substrates other than ammonia could limit the distribution of some environmental populations. The capacity for strain PS0 to grow at a very low pH suggests that ongoing ocean acidification, rather than limiting the growth of ammonia oxidizers, may actually stimulate some populations and, as a consequence, alter the marine nitrogen cycle in unexpected ways. Finally, since these studies encompassed only a small and genetically closely circumscribed set of AOA, relative to the much greater genetic diversity revealed by ongoing metagenomic surveys, they point to a rich and mostly unexplored physiological diversity of the marine AOA.

Materials and Methods

Enrichment and Isolation of marine AOA. Low added ammonia (2 μM) was used to selectively enrich AOA from seawater samples collected from the Puget Sound estuary in Washington State (*SI Materials and Methods*). Pure cultures were obtained by filtration of the enrichment cultures through a syringe filter, and diluting the filtrate to extinction. For details, see *SI Materials and Methods*.

Growth experiments. All materials and methods for physiology experiments are described in detail in supplementary information.

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Transmission Electron Microscopy. Preparation of the novel AOA isolates for examination by transmission electron microscopy is described in supplementary information.

Sequencing and Phylogenetic Analysis. Sequences of AOA 16S rRNA and *amoA* genes were obtained using an ABI 3730xl sequencer. Phylogenetic trees were generated using MEGA5 as described in *SI Materials and Methods*.

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Figures

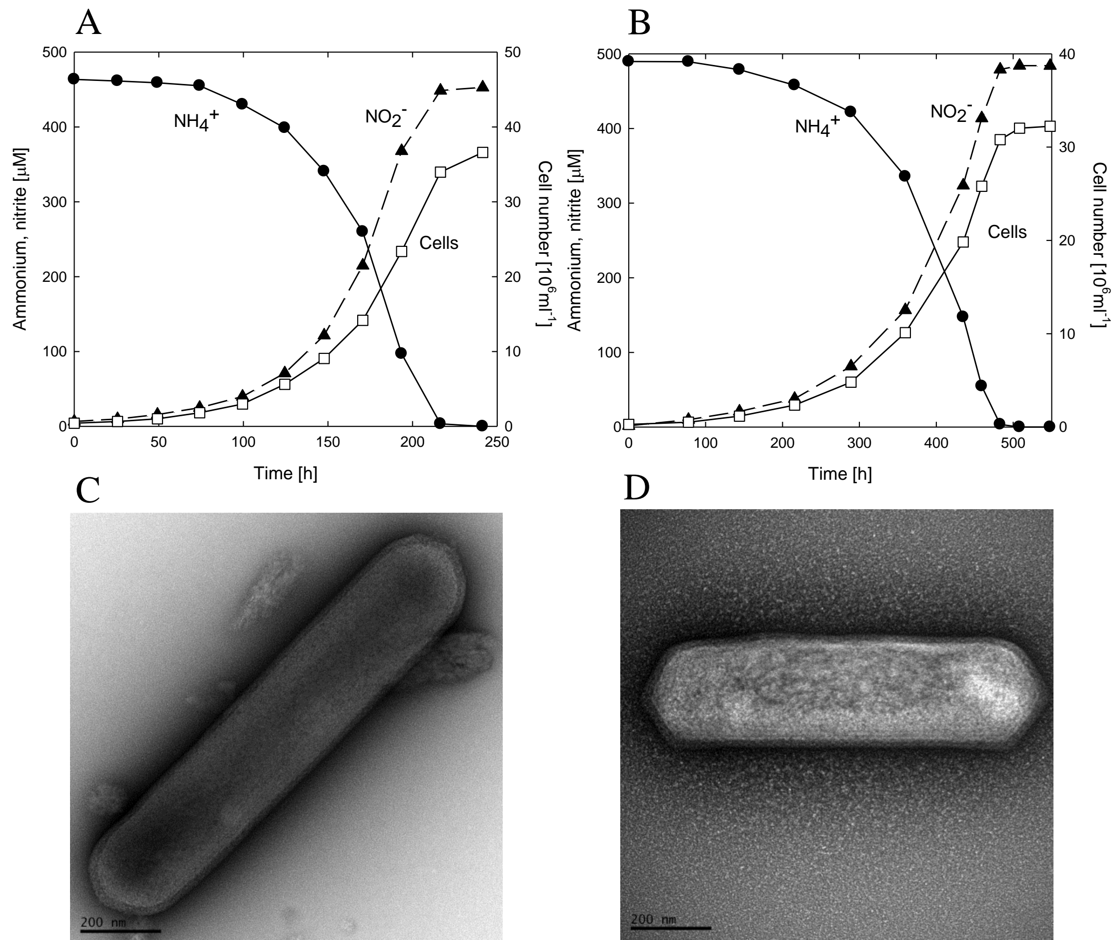

Figure 1. Growth and morphology of strains HCA1 and PS0. Correlation between ammonia oxidation and growth of HCA1 (*A*) and PS0 (*B*) in artificial sea water media containing 500 µM NH_4^+ and 100 µM α-ketoglutaric acid. Transmission electron micrographs of negative-stained cells of HCA1 (*C*) and PS0 (*D*). Scale bars equal 200 nm.

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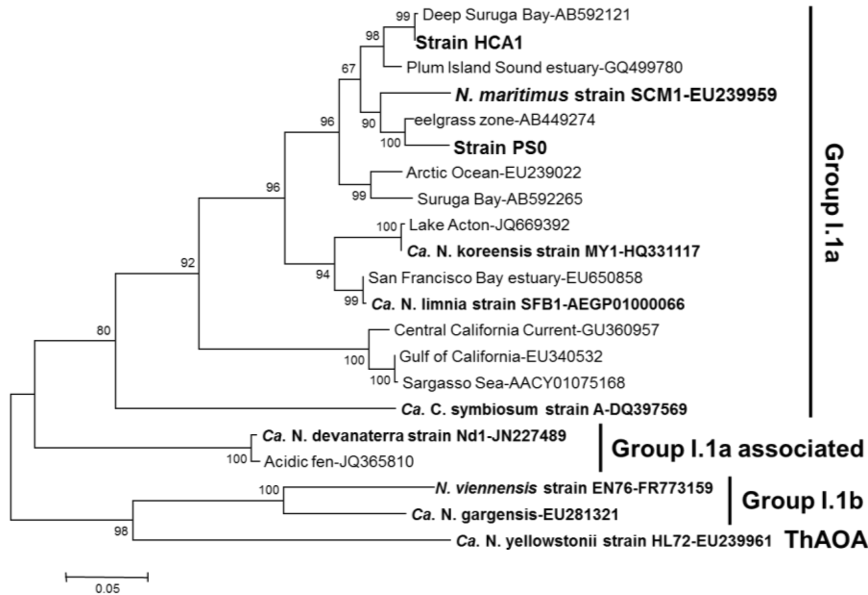

Figure 2. Phylogenetic relationships between *amoA* gene sequences of strains HCA1, PS0, and SCM1 and described AOA representatives, as well as relevant environmental clone sequences. The tree was constructed using the maximum-likelihood method with Kimura 2-parameter correction. Confidence values were based on 1000 bootstrap replications. Scale bar represents 0.05 nucleotide changes per position.

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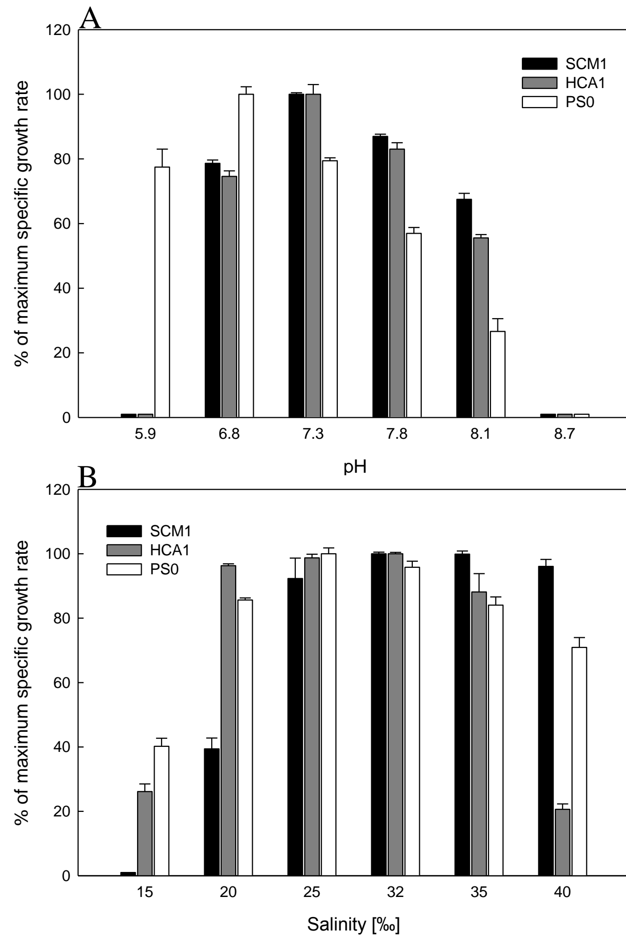

Figure 3. Influence of pH (*A*) and salinity (*B*) on growth. Values represent % of specific growth rates of cultures grown under different pH and salinity values relative to those at pH and salinity optima. Error bars represent the standard deviation of triplicate cultures.

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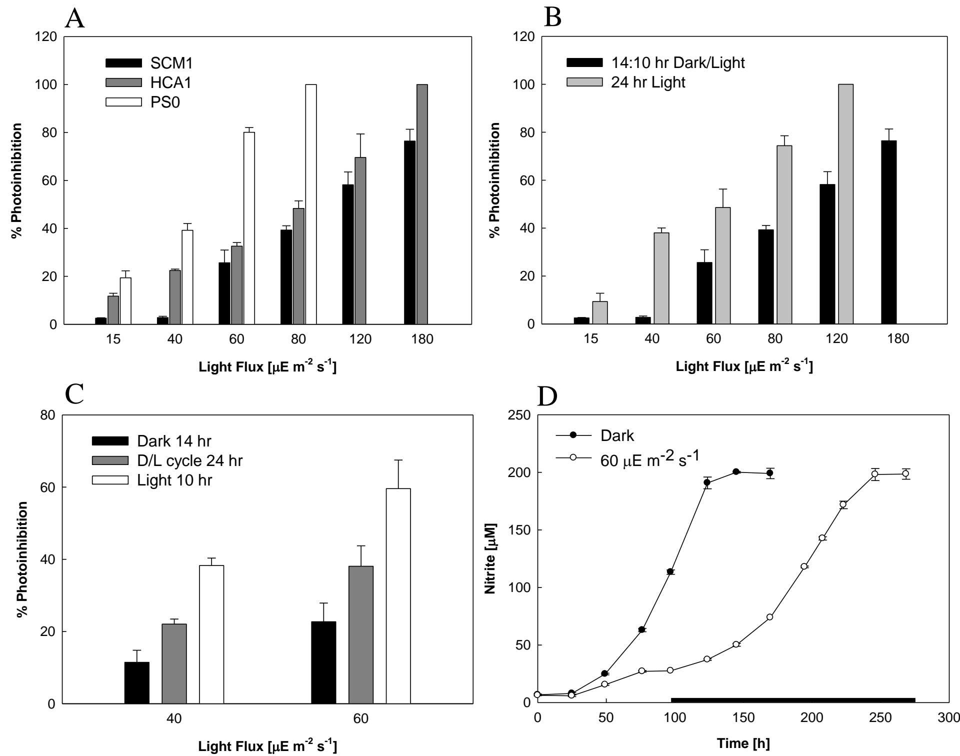

Figure 4. Photoinhibition and recovery of ammonia oxidation of the three AOA strains. Values represent % inhibition of cultures grown under continuous illumination or dark/light cycles relative to cultures grown in the dark. *(A)* The inhibitory effects of light on all three isolates under a 14:10 hour dark/light cycle at different intensities from 15 to 180 μE m^{-2} s^{-1}. (*B*) Photoinhibition of strain SCM1 under continuous light (15 to 120 μE m^{-2} s^{-1}) and dark/light cycles (15 to 180 μE m^{-2} s^{-1}). (*C*) The reduction in specific growth rate of strain HCA1 with cycling between periods of 14 hour dark and 10 hour light at 40 and 60 μE m^{-2} s^{-1}. (*D*) The recovery of light-inhibited SCM1 cultures (open circles) following transfer to the dark after 100 hours of continuous illumination (60 μE m^{-2} s^{-1}) compared to continuous growth in the dark (dark circles). Error bars represent the standard deviation of triplicate incubations.

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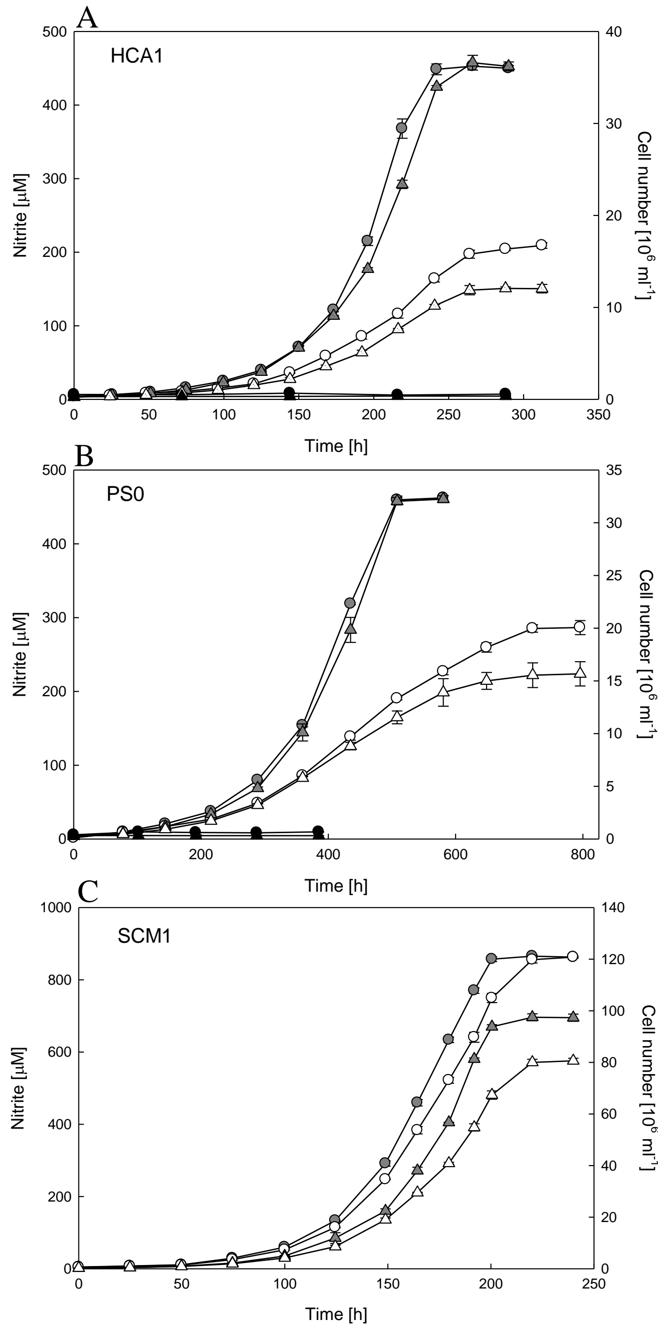

Figure 5. Nitrite production (circles) and growth curves (triangles) of strains HCA1 (*A*), PS0 (*B*), and SCM1 (*C*) with 100 µM α-ketoglutaric acid (gray) relative to controls without organic carbon supplements (first transfer: white; second transfer: black). Error bars represent the

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standard deviation of triplicate cultures (Please note that in the most cases the error bars are too small to be visible in the figure).

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Supplementary figures

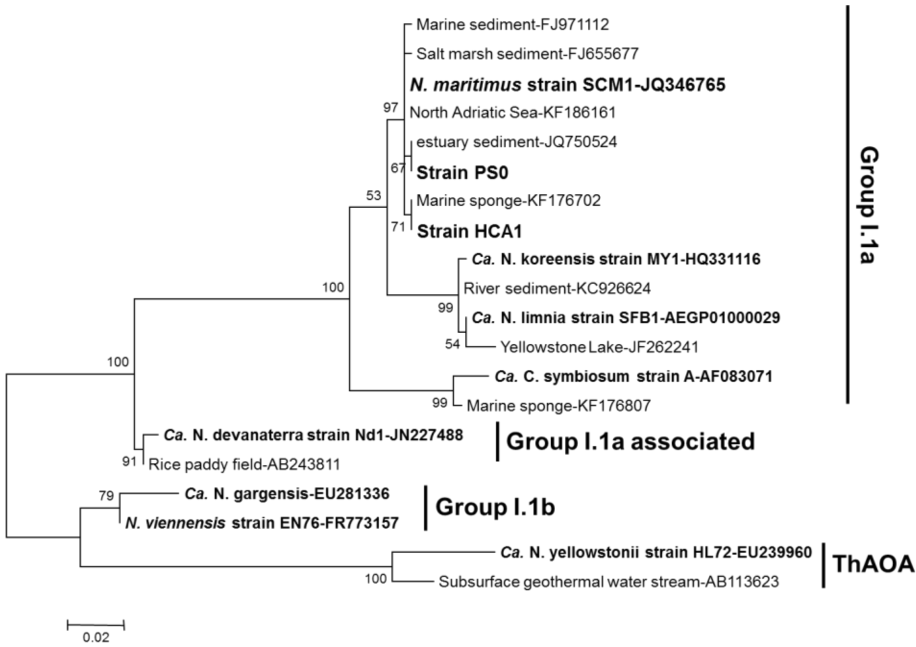

Figure S1. Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences. Confidence values were based on 1000 bootstrap replications. The scale bar represents an estimated sequence divergence of 2%.

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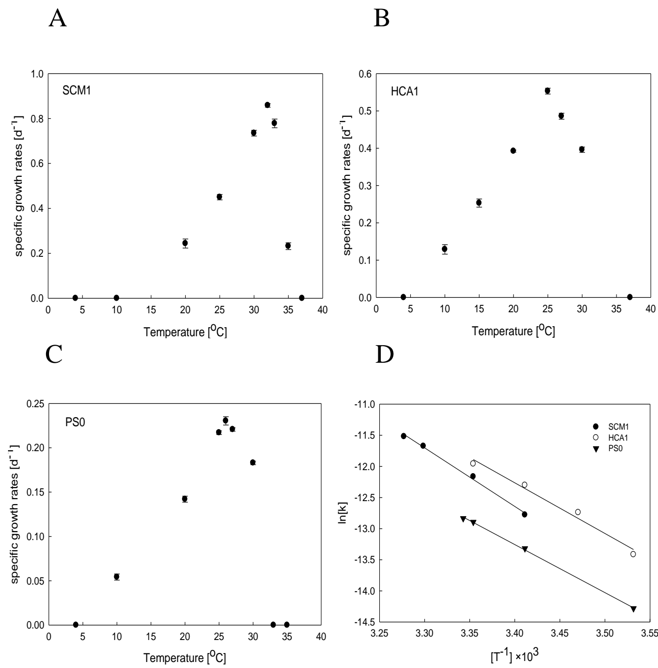

Figure S2. Temperature dependence of the growth of SCM1 (*A*), HCA1 (*B*), and PS0 (*C*). (*D*) Arrhenius analysis showing correlation coefficients of 0.99, 0.98, and 0.99 for strains SCM1, HCA1, and PS0 respectively. Data are presented as the mean of triplicate cultures. The estimated apparent activation energy and Q_{10} values are shown in Table S2.

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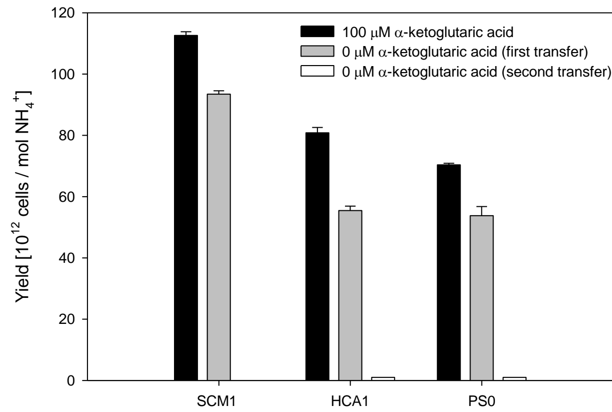

Figure S3. The specific growth yields of cultures of three AOA isolates with 100 µM α-ketoglutaric acid (black) relative to controls without α-ketoglutaric acid (first transfer: gray; second transfer: white). The specific growth yields were calculated from cell numbers. Error bars represent the standard deviation of data from triplicate cultures.

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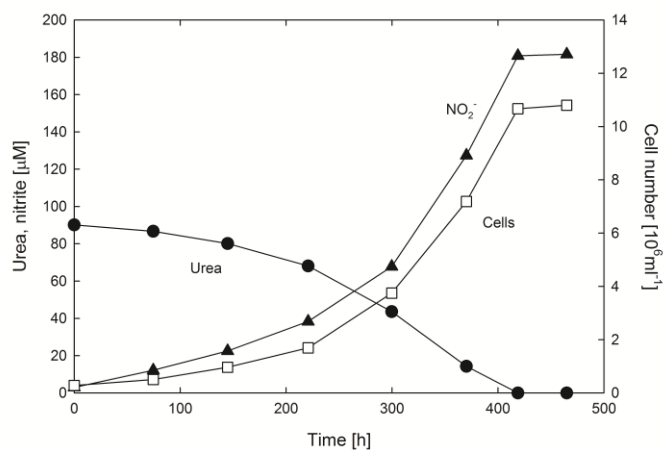

Figure S4. Growth of PS0 in artificial seawater medium containing 90 µM urea, showing near stoichiometric conversion of urea to nitrite. Data points are the mean values of triplicate cultures.

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Supplementary tables

Table 1. Growth kinetics of ammonia oxidation for three marine AOA isolates.

Strain	Maximum specific rates per cell [fmol NO_2^- cell^{-1}d^{-1}]	Maximum specific activities based on biomass [μmol NH_4^+ mg protein^{-1} h^{-1}]
SCM1	12.7	51.9
HCA1	6.0	24.5
PS0	2.9	12.2

Table 2. Activation energy and Q_{10} values of ammonia oxidation for three marine AOA strains and other bacterial ammonia oxidizers.

Strain	Temperature range (˚C)	Activation energy [kJ mol^{-1}]	Q_{10}
SCM1	20 to 32	78.25	2.89
HCA1	10 to 25	67.67	2.62
PS0	10 to 26	64.20	2.49
Estuarine *Nitrosomonas sp.*	10 to 20	82.5	3.3
Estuarine *Nitrobacter sp.*	10 to 20	67.4	2.7

Table 3 Cell yields and growth rates in marine AOA pure cultures with 100μM α-ketoglutaric acid over controls without organic substrate.

Strain	α-ketoglutaric acid [μM]	Maximum Cells density [10^7 cells ml^{-1}]	Specific growth rate [d^{-1}]
SCM1	100	9.7	0.78
	0	8.1	0.76
HCA1	100	3.7	0.55
	0 (first transfer)	1.2	0.43
	0 (second transfer)	0.0	0.00
PS0	100	3.2	0.23
	0 (first transfer)	1.5	0.19
	0 (second transfer)	0.0	0.00

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SI Materials and Methods

Enrichment of marine AOA. Seawater samples were collected from the Puget Sound Regional Synthesis Model (PRISM) Station P10 (47.91 N, 122.62 W) in Hood Canal, and Puget Sound main basin (47.55 N, 122.28W), Washington State, USA. It was previously reported that AOA, rather than AOB, are mainly responsible for nitrification in these regions (Horak et al.; Huguet et al., 2010; Urakawa et al., 2010). The water samples were stored in on-deck incubators maintained at seawater surface temperature in the dark and transferred to the laboratory the same day. A sample volume of 10 ml was supplemented with 2 μM NH_4Cl in a 50 ml polypropylene BD Falcon Tube (BD, Franklin Lakes, NJ, USA) and incubated at 15 ˚C. Cultures were monitored for ammonia consumption and repeatedly subcultured with a 10% inoculum into filtered seawater from the two stations (0.22 μm STERIVEX- GP filter unit, Millipore) under the same enrichment conditions. Antibiotics were not applied for the initial enrichment to avoid the selection of antibiotic-resistant bacteria. Following reproducible growth in the filtered seawater media, enrichments of PS0 and HCA1 were then maintained in artificial seawater medium with 2 μM NH_4Cl and 100 μM α-ketoglutaric acid. Synthetic seawater was autoclaved (Konneke et al., 2005), cooled to room temperature, and the following components added from sterile stock solutions: 3.0 ml 1 M sodium bicarbonate, 5.0 ml 0.4 g l^{-1} KH_2PO_4**,** 1ml 7.5 mM FeNaEDTA, 1ml non-chelated trace element mixture (Martens-Habbena et al., 2009), and 2 μl 1M NH_4Cl. None of enrichments survived in the synthetic medium without α-ketoglutaric acid. Two highly enriched cultures were obtained after more than two years of low ammonium selection (2 μM NH_4Cl).

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Isolation of marine AOA. Growth rates of cultures supplemented with 50 µM instead of only 2 µM NH_4Cl were comparable, hence, 50 µM ammonia was used to further isolate marine AOA in bicarbonate-buffered synthetic marine Crenarchaeota medium. The cultures were additionally supplemented with 100 µM α-ketoglutaric acid and 40 mg l^{-1} streptomycin and incubated at 20 °C in the dark without shaking. Pure cultures were obtained by filtering exponential phase enrichment cultures through 0.22 µm Millex-GP syringe filters, followed by repeated end-point dilution in streptomycin and α-ketoglutaric acid supplemented medium. The absence of bacterial contaminants was checked by PCR, showing no amplification with general primers for the bacterial 16S rRNA gene (Lane, 1991) or with those specific for the *amoA* gene of ammonia-oxidizing bacteria (Rotthauwe et al., 1997; Purkhold et al., 2000). The purity of AOA cultures was routinely monitored by microscopic inspection and by absence of bacterial growth in marine broth medium (5 g peptone and 1 g yeast extract, 250 ml Milli-Q water, and 750 ml autoclaved seawater).

Growth experiments. Strains PS0 and HCA1 were transferred from bicarbonate-buffered medium (see above) to HEPES-buffered synthetic Crenarchaeota medium (Martens-Habbena et al., 2009) supplemented with 500 µM NH_4Cl and 100 µM α-ketoglutaric acid. Since no difference in growth rate was observed for the HEPES- and bicarbonate-buffered media, unless otherwise indicated, the growth response of each strain to varying temperature, salinity, pH, and light was determined in triplicate 100 or 500 ml cultures using the HEPES-buffered medium. The pH of the medium was adjusted to a range between 5.9 and 8.7 using a pH meter (Orion model 420, Thermo Scientific, Waltham, MA, USA) for initial titration with either 12.1 M HCl or 5 M KOH, followed by verification of pH values in the range of 7 to 9 using the indicator dye

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m-cresol purple before inoculation (Dickson, 2009). No, or only a negligible, decrease in pH could be detected between the beginning and end of each growth experiment. To assess the influence of salinity on growth, a stock solution of artificial seawater at 40‰ was prepared containing NaCl (32.5 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (6.25 g l^{-1}), $MgCl_2 \cdot 6H_2O$ (6.25 g l^{-1}), $CaCl_2 \cdot 2H_2O$ (1.875 g l^{-1}) and KBr (0.125 g l^{-1}), and portions of it taken and diluted with Milli-Q water to 15 to 35‰. To investigate the inhibitory effect of light, cultures were grown in glass bottles that were either kept in the dark or exposed to cool white fluorescent lamps (Phillips F17T8/TL741, 17W) with wavelength output ranging from 410-720 nm (Tong et al., 2008). Light intensities measured inside and outside the growth bottle with a Biospherical Instruments QSL-2100 radiometer (San Diego, CA) showed less than 10% attenuation of light by the glass. For growth experiments examining the utilization of urea as an energy source, the background ammonium concentration in the urea medium was less than 1µM and thus did not represent a major source of energy.

Activation energy and Q_{10}. Activation energies were calculated based on the slopes of linear regression lines of Arrhenius plots. Q_{10} values between the minimum growth temperature and temperature optimum were obtained using:

$$Q_{10} = \exp\left[\frac{E_a \cdot 10}{RT(T + 10)}\right]$$

Here E_a is the activation energy (J mol^{-1}), R is the molecular gas constant (8.314 J K^{-1} mol^{-1}), and T is the absolute temperature (K).

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Growth Parameters Measurements. Ammonia concentration was measured by the o-phthaldialdehyde fluorescence method (Holmes et al., 1999) and a fluorescence microplate reader (Infinite F500, TECAN, USA) (standard concentration range: 0-1000 µM, n=5 concentrations, R^2>0.997). The concentration of nitrite and urea were determined spectrophotometrically using the Griess reagent (Stickland) (standard concentration range: 0-1000 µM, n=6 concentrations, R^2>0.999) and diacetyl monoxime (Grasshoff, 1983) (standard concentration range: 0-500 µM, n=6 concentrations, R^2>0.999), respectively. Total cell counts were determined using moviol-SybrGreen I staining protocol (Lunau et al., 2005). Briefly, the mounting medium was freshly prepared by adding 3 µl of SybrGreen I stock solution (Invitrogen, Carlsbad, USA) and 2 µl of 1 M ascorbic acid solution into 200 µl of the moviol solution (moviol 4-88, Fluka, Switzerland). Cells were filtered on a 0.02-µm Anopore membrane filter (Whatman, Germany), and then stained with 20 µl of mixed microscopic mounting medium. Cell numbers were determined using a Zeiss Axioskop 2 MOT epifluorescence microscope to count 20 random fields of view for each sample with 10 to 100 cells per field.

Transmission Electron Microscopy. Cells of strains HCA1 and PS0 were fixed overnight with half-strength Karnovsky`s fixative (Karnovsk.Mj, 1965), transferred to a 200-mesh formvar-coated copper grid for 30 min, and then washed successively in a small volume of fresh 0.1 M cacodylate buffer and water. Staining of cells was performed by swirling the grid in a drop of 1% uranyl acetate. Liquid was drained off with filter paper and the grid dried overnight in a desiccator. Each preparation was examined with a JEOL JEM-1400 transmission electron microscope at an accelerating voltage of 120 KV and magnification of 10,000 and 20,000X (Electron Microscopy Core Facility, Fred Hutchinson Cancer Research Center, Seattle, WA).

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Sequencing and Phylogenetic Analysis of 16S rRNA and *amoA* Genes. Cells were collected by vacuum filtration on 0.22-µm Sterivex-GP filters (Millipore Corporation, MA, USA). DNA extracts were obtained as previously described using modified phenol-chloroform method (Urakawa et al., 2010). The AOA 16S rRNA and *amoA* genes were PCR amplified using archaeal-specific primers Arch21F/Arch958R (Delong, 1992) and CrenAmoAModF/CrenAmoAModR (Mincer et al., 2007), respectively, with the protocols described in the original papers (Delong, 1992; Mincer et al., 2007). Amplified products were purified with MinElute PCR Purification kit (Qiagen, Maryland, USA) and cloned using a TOPO TA PCR Cloning kit (Invitrogen). Sequences were obtained using an ABI 3730xl sequencer (Applied Biosystems System operated by the High-Throughput Genomics Center, Seattle, WA). The 16S rRNA and *amoA* genes sequences of strain HCA1 and PS0 were deposited in GenBank under accession numbers KF957663, KF957664, KF957665, and KF957666. The DNA sequences of 16S rRNA and *amoA* genes were aligned with CLUSTAL W program in MEGA5 software and the phylogenetic relationships were analyzed by maximum-likelihood method with Kimura 2-parameter correction using MEGA5. Bootstrap support was based on 1000 iterations for each analysis.

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