LETTERS

Isolation of an autotrophic ammonia-oxidizing marine archaeon

Martin Könneke[1*†], Anne E. Bernhard[1*†], José R. de la Torre[1*], Christopher B. Walker[1], John B. Waterbury[2] & David A. Stahl[1]

For years, microbiologists characterized the Archaea as obligate extremophiles that thrive in environments too harsh for other organisms. The limited physiological diversity among cultivated Archaea suggested that these organisms were metabolically constrained to a few environmental niches. For instance, all Crenarchaeota that are currently cultivated are sulphur-metabolizing thermophiles[1]. However, landmark studies using cultivation-independent methods uncovered vast numbers of Crenarchaeota in cold oxic ocean waters[2,3]. Subsequent molecular surveys demonstrated the ubiquity of these low-temperature Crenarchaeota in aquatic and terrestrial environments[4]. The numerical dominance of marine Crenarchaeota—estimated at 10^{28} cells in the world's oceans[5]—suggests that they have a major role in global biogeochemical cycles. Indeed, isotopic analyses of marine crenarchaeal lipids suggest that these planktonic Archaea fix inorganic carbon[6]. Here we report the isolation of a marine crenarchaeote that grows chemolithoautotrophically by aerobically oxidizing ammonia to nitrite—the first observation of nitrification in the Archaea. The autotrophic metabolism of this isolate, and its close phylogenetic relationship to environmental marine crenarchaeal sequences, suggests that nitrifying marine Crenarchaeota may be important to global carbon and nitrogen cycles.

Since their discovery by Fuhrman *et al.* and DeLong over a decade ago[2,3], marine Crenarchaeota are now recognized to be a dominant fraction of bacterioplankton in the ocean. These microorganisms can account for up to 40% of the bacterioplankton in deep ocean waters[5]. Although there are no known low-temperature Crenarchaeota in culture, compound-specific $\Delta^{14}C$ analysis of lipid biomarkers[6] and studies of ^{13}C-bicarbonate tracer uptake by natural populations[7] have suggested autotrophy. However, another study of natural populations demonstrated the uptake of tracer levels of tritiated amino acids, suggesting some use of fixed carbon[8]. We expect that the availability of a representative organism in pure culture will facilitate studies of their physiology and evolutionary origin, and help in understanding their contribution to oceanic biogeochemical cycles.

Known nitrifying bacteria fall into two distinct physiological groups: those that oxidize ammonia to nitrite, and others that oxidize nitrite to nitrate[9]. None has been shown to oxidize ammonia completely to nitrate. Existing genera of ammonia-oxidizing bacteria (AOB) fall within the Betaproteobacteria and the Gammaproteobacteria (ref. 9). Molecular studies of AOB in nitrifying systems, including aquaria[10], have been limited to these two phylogenetic groups[9]. We first suspected an involvement of Archaea in nitrification after we completed several cultivation-independent ribosomal RNA gene surveys of nitrifying environments. We detected sequences affiliated with the marine group 1 Crenarchaeota in nitrifying dilution cultures developed from Plum Island Sound (Massachusetts) estuary sediment, in nitrifying filtration systems at the Shedd Aquarium (Chicago, Illinois), and in gravel from a marine tropical fish tank at the Seattle Aquarium (Seattle, Washington).

Further evidence for archaeal nitrifiers resulted from ammonia-oxidizing cultures highly enriched in marine group 1 Crenarchaeota. Filtered aquarium water (0.2-μm polyethersulphone membrane; Nalgene) supplemented with 1 mM ammonium chloride was inoculated with gravel from a tropical marine tank at the Seattle Aquarium. Cultures enriched for Crenarchaeota were incubated at 21–23 °C in the dark. Repeated serial transfers of 10% of the culture volume into fresh aquarium-water medium resulted in an enrichment comprised of approximately 90% Crenarchaeota and 10% organisms affiliated with the bacterial domain after six months (data not shown). Characterization of this highly enriched culture revealed that oxidation rates of ammonia to nitrite corresponded with increasing abundance of Crenarchaeota (measured by quantitative polymerase chain reaction (PCR); Supplementary Information) indicating nitrification (data not shown).

After initial enrichment, the Crenarchaeota were isolated in a defined medium (see Methods) containing bicarbonate and ammonia as the sole carbon and energy sources, suggesting autotrophy. A pure culture of Crenarchaeota (designated SCM1) was recovered after three serial end-point dilutions in this medium, facilitated by the addition of streptomycin and filtration of the inoculum through a 0.45-μm HT Tuffryn membrane syringe filter (Pall). The purity of SCM1 was confirmed by quantitative PCR and fluorescent *in situ* hybridization (FISH), and supported by a failure to recover bacterial 16S rRNA genes by PCR amplification or to promote the growth of heterotrophic bacteria by the addition of yeast extract and peptone to the defined culture medium (data not shown). PCR amplification of nearly full-length 16S rRNA genes from SCM1 identified only crenarchaeal sequences. The clonal structure of SCM1 was confirmed by comparing the sequences of PCR-amplified fragments of 1,650 base pairs (bp) in length and containing most of the 16S rRNA, the complete 16S–23S internal transcribed spacer, and a small portion of the 23S rRNA gene (Supplementary Information).

Comparative sequence analysis of 16S rRNA genes revealed a high level of sequence identity (>98%) between SCM1 and marine group 1 Crenarchaeota sequences recovered from the North Atlantic, the Red Sea, the Antarctic and hydrothermal vents (Fig. 1). Phylogenetic analysis indicates that all marine group 1 Crenarchaeota—including SCM1, crenarchaeal sequences from the Sargasso Sea[11] and *Cenarchaeum symbiosum* (an uncultured marine sponge symbiont)[12]—form a monophyletic clade sharing >94% rRNA sequence identity (Fig. 1).

[1]Department of Civil and Environmental Engineering, University of Washington, Seattle, Washington 98195, USA. [2]Woods Hole Oceanographic Institute, Woods Hole, Massachusetts 02543, USA. †Present addresses: Institut für Chemie und Biologie des Meeres, Universität Oldenburg, Oldenburg 26111, Germany (M.K.); Department of Biology, Connecticut College, New London, Connecticut 06320, USA (A.E.B.).
*These authors contributed equally to this work.

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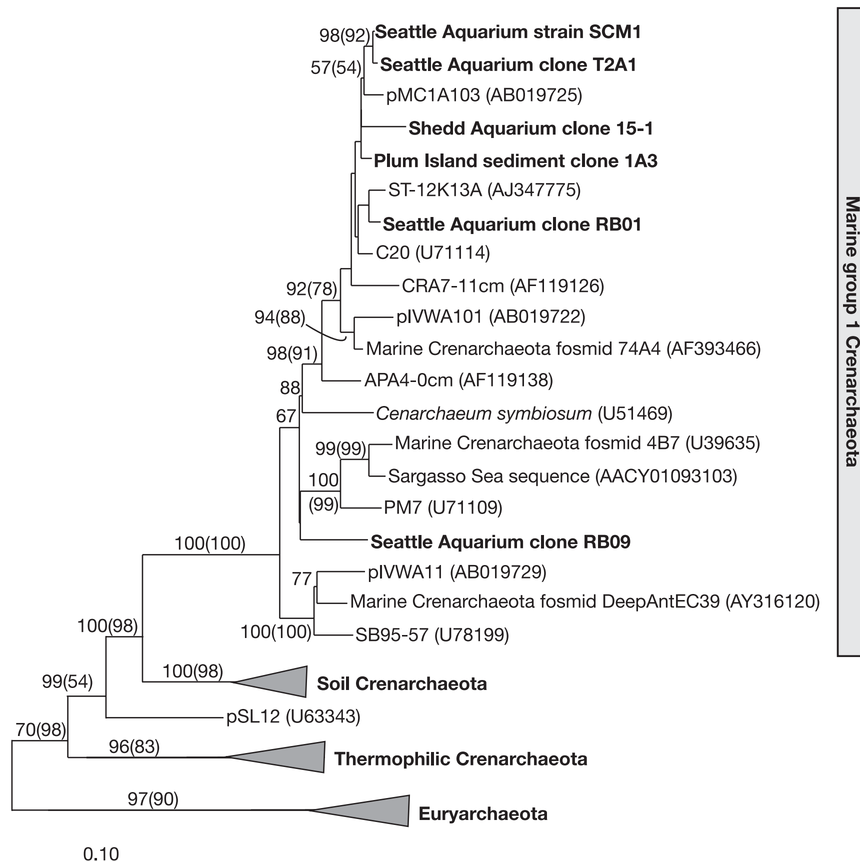


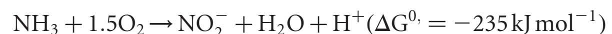
Figure 1 | Phylogenetic relationships between 16S rRNA sequences from SCM1 and representatives of the marine group 1 Crenarchaeota. The tree was constructed using the neighbour-joining algorithm with the Kimura two-parameter correction (1,218 positions). Nodes supported by bootstrap values >50% by neighbour-joining and parsimony (in parentheses) are indicated. The sequences from the Shedd Aquarium and Plum Island sediment clones are short (674 and 720 bp, respectively) and were added to the tree using the parsimony tool in ARB (ref. 25). The scale bar represents 0.1 nucleotide changes per position.

In contrast, members of the marine group 1 Crenarchaeota share only 84% 16S rRNA sequence identity with low-temperature Crenarchaeota found in soil, and less than 80% sequence identity with cultivated thermophilic Crenarchaeota. These differences in 16S rRNA sequences are consistent with variations found in genome fragments from marine and soil Crenarchaeota[13,14]. Nevertheless, phylogenetic analyses clearly indicate that all low-temperature Crenarchaeota are more closely related to each other than to their thermophilic relatives (Fig. 1).

Cells of SCM1 visualized by electron microscopy appear as straight rods with a diameter of 0.17–0.22 μm and a length of 0.5–0.9 μm (Fig. 2). Cells occurred individually or in loose aggregates. Neither flagella nor intracellular compartments were apparent by electron microscopy. The size and morphology of SCM1 cells are nearly identical to marine Crenarchaeota in natural samples visualized by FISH[12,15]. Individual SCM1 cells stained with 16S rRNA polyribonucleotide probes showed strong fluorescence at both poles and weak staining in the central region corresponding to the location of the nucleoid, identified by staining with the fluorescent DNA-binding dye 4′,6′-diamidino-2-phenylindole (DAPI) (Fig. 2a and b; arrows), giving labelled cells the characteristic peanut-like shape previously reported for marine Crenarchaeota[12,15].

SCM1 grew to a maximal density of 1.4×10^7 cells ml^{-1} at 28 °C in defined medium containing 500 μM ammonium, with a minimum generation time of 21 h (Fig. 3). This cell density is approximately three orders of magnitude greater than that observed for marine Crenarchaeota in natural bacterioplankton samples[5]. Ammonium typically reaches concentrations of <0.03–1 μM in the open ocean and <0.03–100 μM in coastal waters[16]. Although this may ultimately limit growth in the marine environment, it is possible that the marine Crenarchaeota are responsible for keeping ammonia concentrations low. The maximum growth rate of SCM1 in culture ($0.78\,d^{-1}$) was somewhat higher than the range of rates estimated for natural bacterioplankton communities, which vary between 0.05 and $0.3\,d^{-1}$ (ref. 17). Notably, the addition of organic compounds, even in very low concentrations, appeared to inhibit the growth of SCM1 (data not shown). Thus, organic material excreted by other organisms (for example, phototrophic primary producers) and a low concentration of ammonium may limit the abundance of marine Crenarchaeota in the environment.

Growth of SCM1 is correlated with near-stoichiometric conversion of ammonia to nitrite (Fig. 3). Among characterized AOB, ammonia monooxygenase (AMO) oxidizes ammonia to hydroxylamine, which is further oxidized to nitrite by hydroxylamine oxidoreductase[9]. The overall reaction is represented by the following stoichiometry:



$$NH_3 + 1.5O_2 \rightarrow NO_2^- + H_2O + H^+ (\Delta G^{0\prime} = -235\,kJ\,mol^{-1})$$

Recently, AMO-related genes have been reported in environmental sequences of marine Crenarchaeota from the Sargasso Sea[11]. Using comparisons of these genes to genome fragments from soil Crenarchaeota[14] (Supplementary Data), we designed oligonucleotide primers to amplify and clone orthologues of the putative A, B and C subunits of AMO from SCM1. The predicted amino acid sequences of the putative AMO-encoding genes from SCM1 are very similar to sequences from Sargasso Sea and soil Crenarchaeota (93–98% and 80–90% amino acid sequence similarity, respectively; Supplementary Information), and are of low similarity to bacterial AMO-encoding

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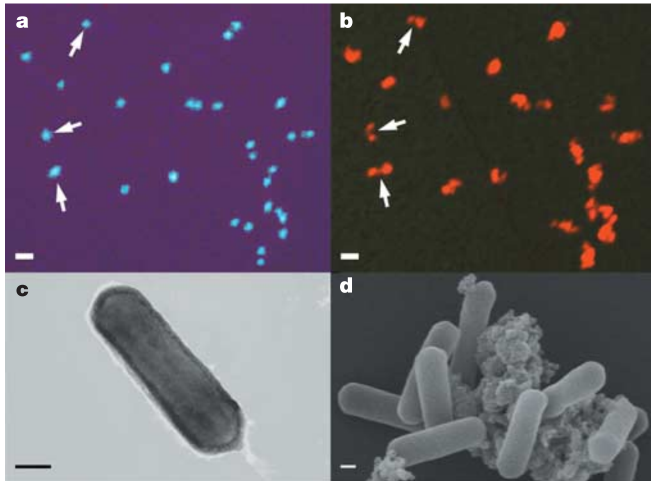

Figure 2 | Photomicrographs of SCM1. a, b, Fluorescence image of cells in identical fields of view stained with DAPI (**a**) and after hybridization with nucleotide polyprobes targeting SCM1 cells (**b**). Arrows indicate cells showing the characteristic peanut-like shape of marine Crenarchaeota[12,15]. Scale bars represent 1 μm. **c**, Transmission electron micrograph of negative-stained cells. Scale bar represents 0.1 μm. **d**, Scanning electron micrograph of Au/Pd-sputtered cells. Scale bar represents 0.1 μm.

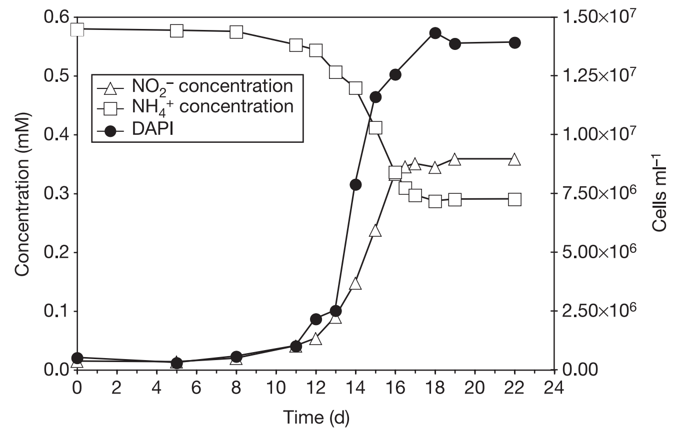

Figure 3 | Near-stoichiometric conversion of ammonia to nitrite by SCM1. Growth of SCM1 in Synthetic Crenarchaeota Media containing ammonium chloride and bicarbonate as sole energy and carbon sources, respectively. DAPI-stained cells were directly counted on filters by fluorescence microscopy. Ammonium consumption and nitrite production were determined in triplicate as described previously[27].

genes (38–51% amino acid sequence similarity). Although it remains uncertain whether these archaeal genes are orthologues of bacterial AMO-encoding genes, our studies correlate for the first time the presence of these archaeal AMO-encoding genes with nitrification. The presence of putative AMO-encoding genes in divergent marine and soil Crenarchaeota implies a broad distribution of nitrifying physiology in these organisms.

SCM1 oxidizes ammonia in the absence of organic carbon, conclusively demonstrating that SCM1 fixes inorganic carbon, a trait common to all known nitrifiers[9]. Isotopic analyses of membrane lipids extracted from the environment implicate the 3-hydroxypropionate carbon-fixation pathway in marine Crenarchaeota[6]. This pathway also exists in thermophilic Crenarchaeota such as *Acidianus infernus*, *Sulfolobus metallicus* and *Metallosphaera medulla*[18]. However, as other carbon fixation pathways also exist in these organisms[19], the elucidation of the pathway(s) associated with mesophilic Crenarchaeota will require further investigation.

SCM1 is the first reported chemolithoautotrophic nitrifier in the domain Archaea and the first mesophilic isolate within the phylum Crenarchaeota. We propose the following candidate status:

Nitrosopumilales order nov.

Nitrosopumilaceae fam. nov.

'*Nitrosopumilus maritimus*' gen. et sp. nov.

Etymology. *nitrosus* (Latin masculine adjective): nitrous; *pumilus* (Latin masculine adjective): dwarf; *maritimus* (Latin masculine adjective): belonging to the sea. The name alludes to the habitat and size of the organism, in addition to its ability to convert ammonia to nitrite.

Locality. The rocky substratum of a tropical marine tank at the Seattle Aquarium (Seattle, Washington, USA).

Diagnosis. A chemolithoautotrophic nitrifier of the domain Archaea, appearing as straight rods with a diameter of 0.17–0.22 μm and a length of 0.5–0.9 μm.

Although we acknowledge that '*N. maritimus*' was not isolated directly from the open ocean, its close phylogenetic relationship to the marine group 1 Crenarchaeota, and the high amino acid sequence similarity between putative AMO-encoding genes from our isolate and from marine crenarchaeal environmental sequences[11], raises the possibility that nitrifying Crenarchaeota contribute to marine carbon and nitrogen cycles. Chemolithoautotrophy enables organisms such as '*N. maritimus*' to inhabit oligotrophic environments where they may function as important primary producers. In environments devoid of organic energy sources and sunlight, the oxidation of ammonia could contribute to primary productivity, and may explain the success of some marine Crenarchaeota in ecological niches such as the deep ocean and polar surface waters during winter[4]. This hypothesis is further supported by the parallels between the depth of the marine nitrite maximum[20] and the vertical distribution of Crenarchaeota in the ocean[5].

Several authors have proposed that the low-temperature Crenarchaeota are derived from a thermophilic lineage and evolved to colonize mesophilic environments[4]. Previous studies of Yellowstone National Park (USA) hot springs have identified crenarchaeal 16S rRNA sequences that are phylogenetically most related to the low temperature Crenarchaeota[21] (clone pSL12, Fig. 1). A recent study has reported the presence of crenarchaeol, a membrane lipid previously thought to be found exclusively in marine Crenarchaeota, in terrestrial hydrothermal springs[22]. High concentrations of bicarbonate were correlated with the abundance of crenarchaeol in these hydrothermal systems. These observations raise the possibility of the existence of thermophilic ammonia-oxidizing Crenarchaeota. Further biochemical and genomic studies of '*N. maritimus*' will provide a foundation for a more complete census of the habitat range of nitrifiers. The discovery of a nitrifying archaeon also highlights the questions of whether ammonia oxidation originated within the bacteria or the archaea, and whether the archetypical nitrifier was a thermophile.

METHODS

Cultivation. Cultures were grown aerobically at 28 °C in Synthetic Crenarchaeota Media containing NaCl (26 g l^{-1}), $MgCl_2{\cdot}6H_2O$ (5 g l^{-1}), $MgSO_4{\cdot}7H_2O$ (5 g l^{-1}), $CaCl_2$ (1.5 g l^{-1}) and KBr (0.1 g l^{-1}). After autoclaving, 1 ml non-chelated trace element mixture[23], 1 ml vitamin solution[23], 10 ml KH_2PO_4 solution (4 g l^{-1}), 1 ml selenite-tungstate solution[23], 1 ml bicarbonate solution (1 M), and 0.5–1 ml ammonium chloride (1 M) were added aseptically per litre of media. The pH was adjusted to 7.0–7.2 using NaOH. Growth was monitored by microscopy, quantitative PCR and nitrite production.

16S rRNA gene sequence and phylogenetic analysis. PCR products amplified with the archaeal-specific primer Arch21F (ref. 3) and the universal primer Univ1492R (ref. 24) were cloned using the TOPO TA Cloning Kit (Invitrogen). Sequences were obtained using ABI 3730XL (Applied Biosystems) or MegaBACE1000 (Amersham) automated DNA sequencers. Phylogenetic relationships were analysed by evolutionary distance and parsimony methods using the ARB software package[25]. Evolutionary distances were calculated with the Kimura two-parameter distance correction. Regions of ambiguity were not included in

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the analysis. Confidence estimates of branching order were determined in PAUP* 4.0 (ref. 26). Short sequences (<1,000 bp) were added to the final tree using the parsimony tool in ARB.

Fluorescence *in situ* hybridization. Fluorescently labelled polyribonucleotide probes targeting crenarchaeal 16S rRNA sequences were synthesized by *in vitro* transcription of nearly full-length crenarchaeal 16S ribosomal DNA clones amplified from enrichment cultures as previously described[15]. Whole-cell hybridizations and calculations of archaeal cell abundances were carried out on cells filtered onto 0.2-μm polycarbonate GTTP membranes (Millipore) following established protocols[15]. The specificity of the labelling was confirmed by the failure to hybridize against *Escherichia coli* and *Desulfovibrio vulgaris* cells.

Transmission electron microscopy. Concentrated cells of SCM1 were fixed with 1% glutaraldehyde. The fixed sample suspension was diluted 1:1 with 2% uranyl acetate in water as droplets on Parafilm. A 200-mesh formvar-coated copper grid was floated on each cell/stain droplet for 5 min. Liquid was drained off with filter paper and the grid was air-dried. Each preparation was examined and photographed with a JEM 1200EXII transmission electron microscope (JEOL) at an accelerating voltage of 80 kV and magnifications of ×30,000 and ×60,000.

Scanning electron microscopy. Glutaraldehyde-fixed cell suspensions were applied to glass coverslips coated with poly-L-lysine, allowed to adhere for 5 min, rinsed with water and post-fixed with 1% OsO_4 for 30 min. Cells on coverslips were then rinsed with water, dehydrated through a graded ethanol series to absolute ethanol, and then critically point dried with L-CO_2. Coverslips were mounted on an aluminium stub, sputter-coated with Au/Pd and examined with a JSM 6300F scanning electron microscope (JEOL) at an accelerating voltage of 15 kV and magnifications of ×20,000–60,000.

Cloning of putative ammonia monooxygenase genes. Putative ammonia monooxygenase gene sequences from soil and planktonic mesophilic Crenarchaeota[11,14] were aligned and used to design degenerate oligonucleotides for PCR amplification of *amoA* (CrenAmo1F, 5′-AATGGTCTGGCTWAGACGC-3′;CrenAmo1R, 5′-GACCARGCGGCCATCCA-3′), *amoB* (CrenAmo2.1F, 5′-CACGGTGTM CAAGCACA-3′; CrenAmo2.2R, 5′-RATTACYTGCCAVGGTC-3′) and *amoC* (CrenAmo3.1F, 5′-ATGGCACARATGCCSGC-3′; CrenAmo3R, 5′-GGTATWG ATCTGCTGTACAA-3′). All PCR reactions were carried out as described for the 16S rRNA genes, except that 2.5 mM $MgCl_2$ was used in the PCR reaction mixture and the template DNA was 50 ng of purified genomic DNA from SCM1. All amplified AMO-encoding genes were cloned and sequenced as described above.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information The sequences described in this manuscript have been deposited in GenBank under accession numbers DQ085097 to DQ085105. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests.
Correspondence and requests for materials should be addressed to D.A.S. (dastahl@u.washington.edu).

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