Environmental Microbiology (2008) **10**(1), 810–818 doi:10.1111/j.1462-2920.2007.01506.x

Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol

José R. de la Torre,[1†] Christopher B. Walker,[1†] Anitra E. Ingalls,[2] Martin Könneke[3] and David A. Stahl[1*]

[1]*Department of Civil and Environmental Engineering, University of Washington, Seattle, WA 98195-2700, USA.*

[2]*School of Oceanography, University of Washington, Seattle, WA 98195-5351, USA.*

[3]*Institut für Chemie und Biologie des Meeres, Universität Oldenburg, Oldenburg 26111, Germany.*

Summary

The widespread occurrence and diversity of ammonia oxidizing *Archaea* suggests their contribution to the nitrogen cycle is of global significance. Their distribution appeared limited to low- and moderate-temperature environments until the recent finding of a diagnostic membrane lipid, crenarchaeol, in terrestrial hot springs. We report here the cultivation of a thermophilic nitrifier ('*Candidatus* Nitrosocaldus yellowstonii'), an autotrophic crenarchaeote growing up to 74°C by aerobic ammonia oxidation. The major core lipid of this archaeon growing at 72°C is crenarchaeol, providing the first direct evidence for its synthesis by a thermophile. These findings greatly extend the upper temperature limit of nitrification and document that the capacity for ammonia oxidation is broadly distributed among the *Crenarchaeota*.

Introduction

The nitrogen cycle has undergone substantial revision with recent discoveries of anaerobic ammonia oxidation (Strous *et al*., 1999) and nitrite oxidation by anoxygenic phototrophs (Griffin *et al*., 2007). The microbiology of this cycle has also been significantly revised with recognition that key processes are more broadly distributed among the primary domains of life than previously appreciated. This now includes nitrogen fixation by hyperthermophilic *Archaea* (Mehta and Baross, 2006), complete denitrification by foraminifera (Risgaard-Petersen *et al*., 2006) and the isolation of aerobic ammonia oxidizing *Archaea* (AOA) (Könneke *et al*., 2005). The discovery of AOA attracted considerable attention as the one available isolate (*Nitrosopumilus maritimus*) is affiliated with a clade of *Crenarchaeota* that comprises nearly 20% of open ocean bacterioplankton (Karner *et al*., 2001; Church *et al*., 2003). The implication that archaeal nitrification is of global significance was consistent with more general environmental surveys of genes encoding the 16S rRNA and a subunit of the putative archaeal ammonia monooxygenase (Francis *et al*., 2005; Treusch *et al*., 2005; Hallam *et al*., 2006). Together, the results of these microbiological and molecular studies served as a basis to infer that AOA are diverse, widely distributed among moderate temperature aquatic and terrestrial environments (Francis *et al*., 2005; Treusch *et al*., 2005; Hallam *et al*., 2006), and often more abundant than bacterial ammonia oxidizers (AOB) (Leininger *et al*., 2006; Wuchter *et al*., 2006; Coolen *et al*., 2007; Lam *et al*., 2007).

Characterization of more extreme habitats colonized by AOA and AOB has included only limited inspection of geothermal environments. There is one report of nitrification at 55°C in a culture containing organisms related to bacterial nitrifiers (Lebedeva *et al*., 2005), and recent molecular surveys of several moderate temperature geothermal systems (45–60°C) reported ribosomal RNA and *amo*-like genes affiliated with archaeal and bacterial nitrifiers (Takai *et al*., 2001; Hirayama *et al*., 2005; Nunoura *et al*., 2005; Spear *et al*., 2007; Weidler *et al*., 2007). The finding of crenarchaeol [a glycerol dialkyl glycerol tetraether (GDGT) membrane core lipid of mesophilic *Crenarchaeota*] in terrestrial hot springs suggested the existence of thermophilic members of this ammonia-oxidizing assemblage (Pearson *et al*., 2004; Zhang *et al*., 2006). Although suggestive, these data alone do not establish the existence of thermophilic ammonia-oxidizing *Archaea* nor unambiguously identify them as a source of crenarchaeol in hot springs (Schouten *et al*., 2007).

We report here the cultivation of a single archaeal population growing autotrophically by aerobic ammonia oxidation at temperatures up to 74°C. This thermophilic crenarchaeote contains *amo*-like genes closely related to those of mesophilic AOA and synthesizes crenarchaeol as its principal membrane core lipid. Ammonia oxidation was correlated directly with archaeal growth and, as with all known nitrifiers, the stoichiometric production of nitrite.

Received 5 October, 2007; accepted 24 October, 2007. *For correspondence. E-mail dastahl@u.washington.edu; Tel. (+1) 206 685 3464; Fax (+1) 206 685 9185. †These authors contributed equally to this work.

Inspection of the Yellowstone National Park (YNP) source feature confirmed the presence of crenarchaeol and nearly identical archaeal rRNA and *amo* gene sequences. Together these data significantly extend the upper temperature limit of nitrification, increase the known diversity and habitat range of ammonia-oxidizing microorganisms and demonstrate the production of crenarchaeol by thermophilic *Archaea*.

Results and discussion

The existence of thermophilic AOA was initially suggested by analysis of genomic DNA and lipids extracted from multiple YNP hot springs spanning a wide range of physical and chemical conditions (Table 1). Archaeal *amoA*-like genes amplified from hot spring environmental DNA formed four relatedness clusters containing 45 novel sequence types (defined by 99% predicted amino acid sequence identity, Fig. 1). Sequences in clusters I and II were recovered from a single sample in the Mammoth Hot Springs area in YNP and affiliated with *amoA*-like sequences previously recovered from other moderately thermal (42–50°C) environments (Spear *et al*., 2007; Weidler *et al*., 2007). Sequences in cluster III were recovered from Octopus Spring and Heart Lake 4 (pH near 8.0 and temperatures above 70°C; Table 1) and were not closely related to any previously published sequence (Fig. 1). Sequences defining cluster IV were recovered from a wide variety of predominantly alkaline hot spring samples (pH 7.2–9.0; one site with pH 3.0) with temperatures from 60°C to 95°C and ammonium concentrations varying from ~100 μM to below detection limit (Table 1). Cluster IV also includes *amoA*-like sequences nearly identical to that of the newly cultivated thermophilic archaeal nitrifier described in this report. All sequences in cluster IV contain a 3 bp insertion absent in all other known *amoA*-like sequences, resulting in a predicted glycine insertion at position 90 of cluster IV predicted protein sequences.

An archaeal contribution to thermophilic nitrification was confirmed by the development of ammonia-oxidizing cultures. A mineral salts medium (Synthetic Freshwater *Crenarchaeota* Medium) containing ammonium chloride (1 mM) and bicarbonate (5 mM) as the sole energy and carbon sources was inoculated with hot spring sediments and incubated at various temperatures (60–80°C) in the dark. Nitrite production was not observed in uninoculated media or at temperatures above 74°C. Stable ammonia-oxidizing enrichments were established at 72°C with samples from six hot springs (Octopus Spring, Witch's Navel and Heart Lake hot springs 1, 3, 4 and 5; Table 1) and maintained for up to 2 years by routine transfer of 10% inoculum into fresh medium. Growth of these thermophilic enrichments was associated with the stoichio-

Table 1. Physical and chemical properties of YNP sampling sites used for the detection of thermophilic AOA. GPS coordinates for these sites are listed in Table S1.

Site	Abbr[a]	Sample type	Temperature (°C)	pH	NH_4^+ (μM)	NO_2^- (μM)	NO_3^- (μM)	NO_2^- production in primary enrichments	*amoA*-like cluster[b]			
									I	II	III	IV
Heart Lake 1	HL1	Sediment	70–80	8.30	95	3	174	+				•
Heart Lake 3	HL3	Sediment	78.9	7.22	b/d	4	b/d	+				•
Heart Lake 4	HL4	Sediment	76.8	8.01	b/d	11	50	+			•	•
Heart Lake 5	HL5	Sediment	82.5–90	6.90	b/d	b/d	36	+				
Witch's Navel	WN	Sediment	69.8	7.62	b/d	16	364	+				
Octopus Spring	OS	Sediment	70–90	8.02	119	1	21	+			•	
O1A	O1a	Microbial mat	72	n/d	n/d	n/d	n/d	n/d				•
Clearwater Spring	CS	Sediment	54	6.2	n/d	n/d	n/d	n/d				•
Mammoth Spring	MamSp	Sediment	n/a	n/d	n/d	n/d	n/d	n/d	•			
Nymph Creek	NyCr	Microbial mat	60	3.00	n/d	n/d	n/d	n/d		•		
Shoshone Spring 7	SHO7	Sediment	70	8.0–9.0	n/d	n/d	n/d	n/d				•
Obsidian Pool Fumarole	OPF	Microbial mat	95	n/d	n/d	n/d	n/d	n/d				•

a. Abbreviations used for clone names in Figs 1 and 3.
b. Sequence clusters (Fig. 1) of archaeal amoA-like genes amplified from environmental samples.
n/a, not available; n/d, data not determined; b/d, below detection limit.

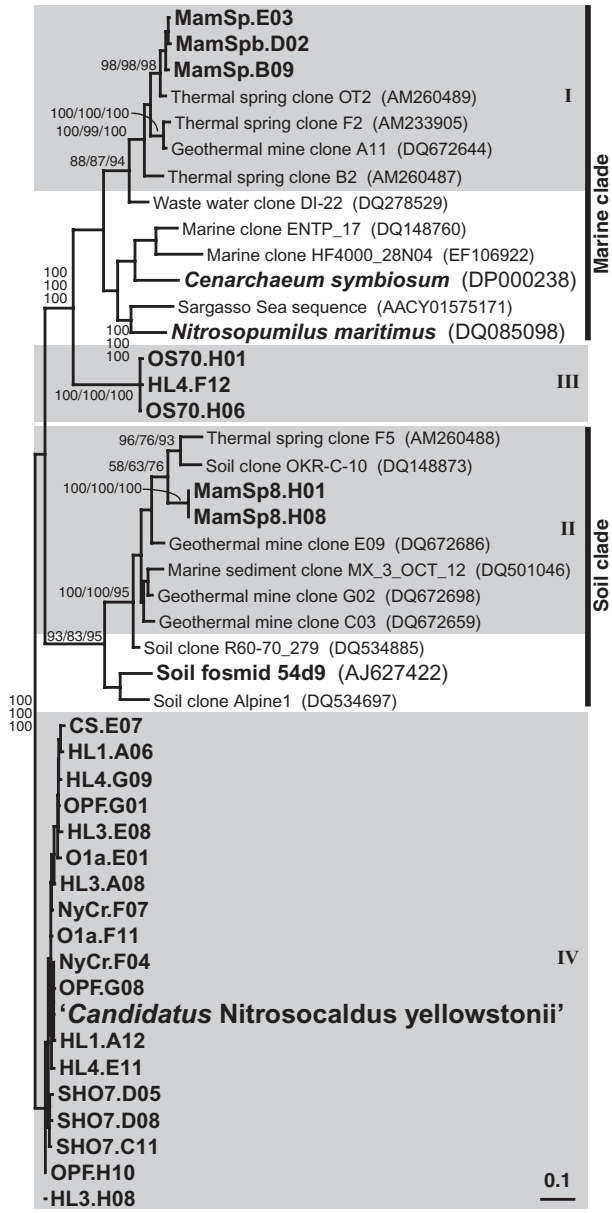


Fig. 1. Phylogenetic analysis of archaeal *amoA*-like nucleotide sequences from '*Candidatus* Nitrosocaldus yellowstonii', YNP hot springs and a variety of mesophilic environments. The tree was constructed using Neighbour-joining inference method with the Kimura 2-parameter correction (598 positions) and shows the relationship between thermophilic *amoA*-like sequences and sequences from mesophilic environments. Thermophilic clusters I, II, III and IV are indicated in grey shading. Nodes supported by bootstrap values > 50% using neighbour-joining (1000 replicates), parsimony (1000 replicates) and maximum likelihood (100 replicates), respectively, are indicated. Scale bar represents 0.1 changes per site. For clone abbreviations, see Table 1.

metric oxidation of ammonia to nitrite, as previously reported for the ammonia oxidizing archaeon *Nitrosopumilus maritimus* (Könneke *et al*., 2005).

The enrichment developed from Heart Lake hot spring 1 (HL72) was more fully characterized. This culture demonstrated the shortest generation time among the enrichment study set ($\mu = 0.8\ d^{-1}$), produced appreciable nitrite between 60°C and 74°C, and had a growth optimum between 65°C and 72°C (Fig. S1). Fluorescence *in situ* hybridization (FISH)-assisted direct cell counts revealed that coccoid *Archaea* accounted for > 90% of cells in HL72 (reaching $\sim 2 \times 10^7$ cells ml^{-1}), with the remaining cells consisting of long, thin rod-shaped bacteria (Fig. 2). Amplification of a unique archaeal 16S rRNA and ITS sequence from HL72 indicated the presence of a single archaeal population affiliated with a clade basal to the radiation of the mesophilic *Crenarchaeota* (Fig. 3). This sequence is nearly identical (> 99.5%) to sequences recovered directly from the Heart Lake 1 hot spring, is closely related to sequences amplified from other thermal environments (Marteinsson *et al*., 2001; Takai *et al*., 2001; Nunoura *et al*., 2005), but is distinct from cultivated hyperthermophiles (Fig. 3). Bacterial 16S rRNA genes amplified from HL72 were closely related (> 98% nucleotide sequence identity) to published sequences belonging to *Thermus*, *Rhodothermus*, *Thermomicrobium* and *Aquificales* spp. These bacterial lineages do not contain any known bacterial nitrifiers. In addition, we could not amplify bacterial *amoA* genes from HL72 using existing primer sets (Rotthauwe *et al*., 1997).

The recovery of a unique archaeal *amoA*-like sequence (cluster IV) from the HL72 culture was also consistent with enrichment of a single archaeon. Amplification of a genome fragment containing archaeal *amoA*- and *amoB*-like genes revealed a genomic

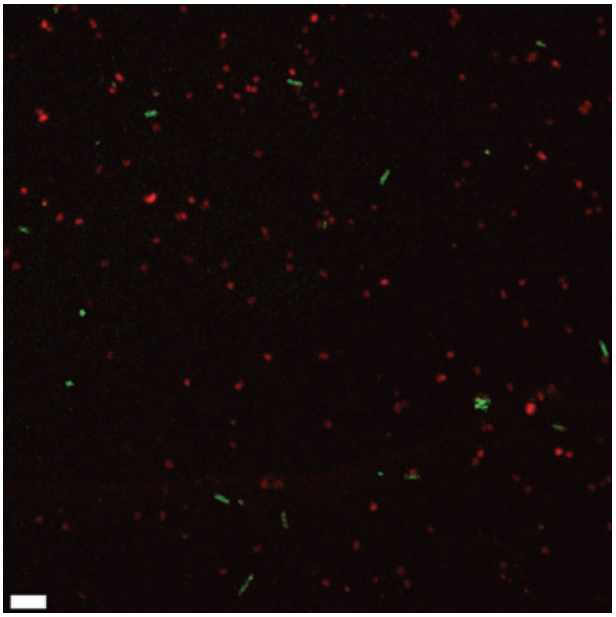


Fig. 2. Epifluorescence photomicrograph of HL72 following FISH to visualize archaeal (red) and bacterial (green) cells. Scale bar corresponds to 5 μm.

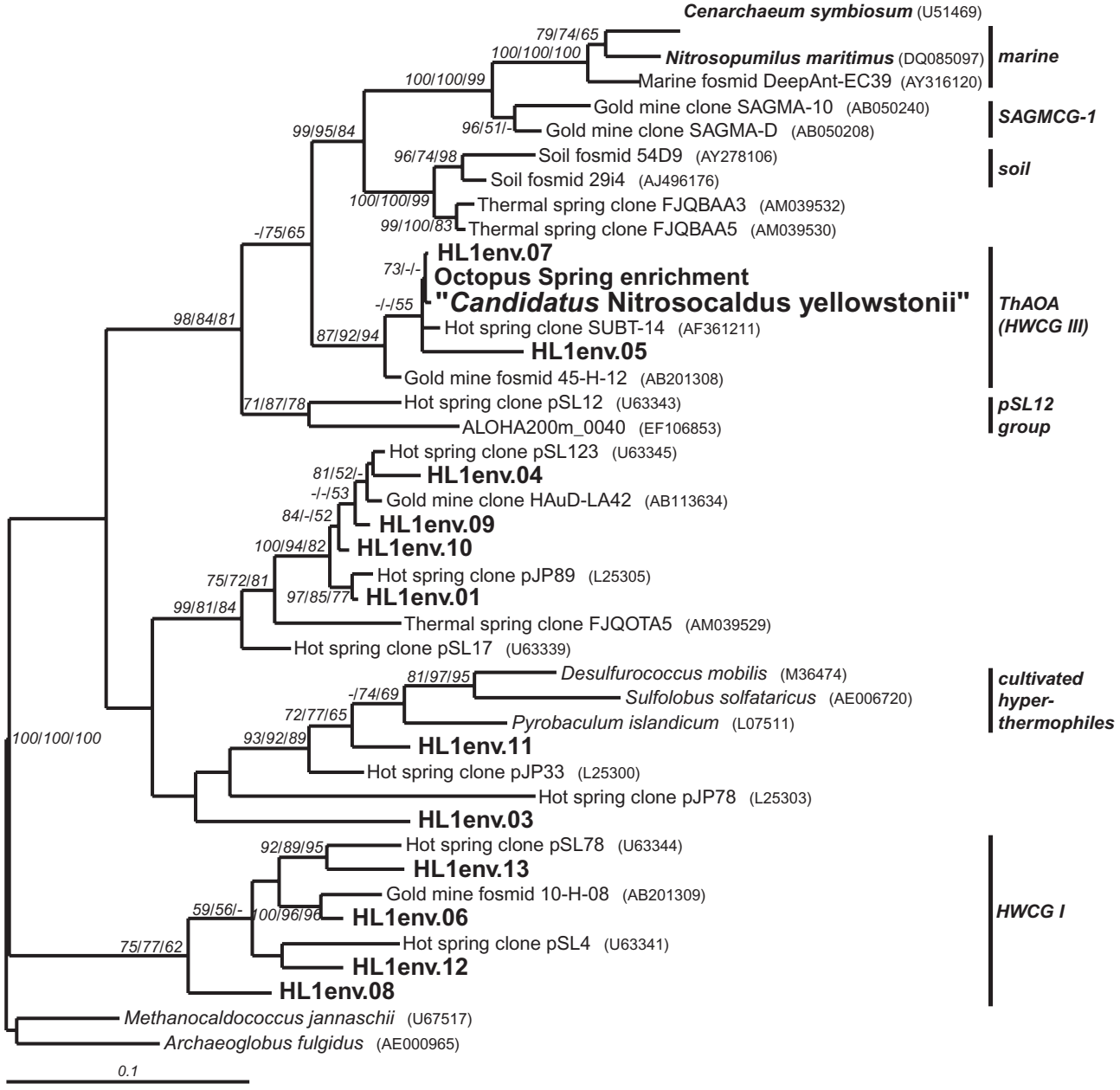


Fig. 3. Phylogenetic analysis of archaeal 16S rRNA gene sequences amplified from '*Candidatus* Nitrosocaldus yellowstonii', YNP hot springs and a variety of mesophilic environments. Neighbour-joining inference method with the Kimura 2-parameter correction (776 positions) illustrates the relationship of '*Candidatus* N. yellowstonii' and Heart Lake 1 archaeal sequences. Nodes supported by bootstrap values > 50% using neighbour-joining (1000 replicates), parsimony (1000 replicates) and maximum likelihood (100 replicates), respectively, are indicated. Scale bar represents 0.1 changes per site. HL1env, Heart Lake 1 environmental clones; SAGMCG-1, South Africa Gold Mine Crenarchaeotic Group 1 (Takai *et al*., 2001); ThAOA, Thermophilic Ammonia-Oxidizing Archaea; HWCG III, Hot Water Crenarchaeotic Group III (Nunoura *et al*., 2005); HWCG I, Hot Water Crenarchaeotic Group I (Marteinsson *et al*., 2001; Takai *et al*., 2001; Nunoura *et al*., 2005).

arrangement similar to the soil *Crenarchaeota* (Fig. S2), lacking a closely linked *amoC*-like gene that is possibly located elsewhere on the chromosome (Nicol and Schleper, 2006). This genomic organization differs from the *amoBCA*-like gene organization observed in the marine *Crenarchaeota* (Hallam *et al*., 2006; Nicol and Schleper, 2006). The predicted amino acid sequence of the HL72 AmoB is highly divergent from all sequences previously recovered from soil and marine habitats (< 50% amino acid sequence identity with a unique 30 amino acid insertion), of possible functional significance for growth at high temperature.

Although multiple isolation strategies (filtration, antibiotics, extinction dilution and flow-cytometry assisted cell

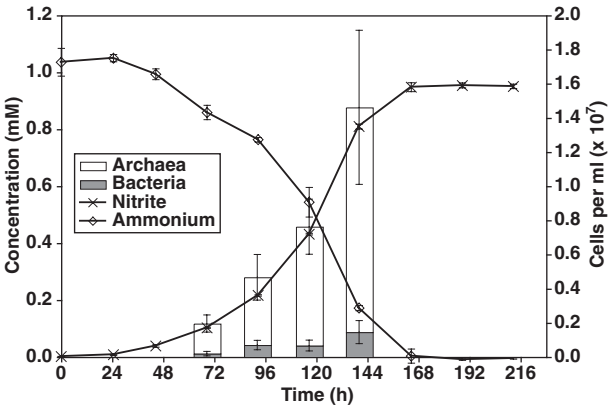


Fig. 4. Correlation of ammonia oxidation with the growth of '*Candidatus* Nitrosocaldus yellowstonii'. Archaeal and bacterial cells were visualized by FISH of filtered culture samples and directly counted by fluorescence microscopy. Measurements of ammonium and nitrite concentrations were determined in triplicate. Error bars represent the standard deviation of the mean.

sorting) have not yielded a pure culture, available data associate the dominant archaeal population with ammonia oxidation. Growth of the archaeon correlated directly with the near-stoichiometric oxidation of ammonia to nitrite (Fig. 4). In contrast, numbers of the minor bacterial population remained relatively constant during the period of exponential nitrite production, only increasing significantly as the culture-approached stationary phase (Fig. 4). Conversion of nitrite to nitrate was not observed. Supplementing the culture medium with dilute yeast extract (0.2 mg l^{-1}), acetate (2 mM) or H_2 (716 torr) reduced and eventually eliminated nitrite production while greatly stimulating bacterial growth (data not shown).

All *Crenarchaeota* examined to date contain GDGT lipids with varying numbers of cyclopentane rings (Fig. S3). Non-thermophilic *Crenarchaeota*, including the proposed ammonia oxidizer *Cenarchaeum symbiosum* (Hallam *et al.*, 2006), have an additional lipid (crenarchaeol) containing four cyclopentane rings as well as a cyclohexane ring (Fig. S3; GDGT-I) (Sinninghe Damsté *et al.*, 2002a,b). Although the ecological and evolutionary significance of crenarchaeol remains unresolved, a broad environmental distribution and correlation with the potential for ammonia oxidation supports its importance as a biomarker (Schouten *et al.*, 2000; 2007; Sinninghe Damsté *et al.*, 2002a; Pearson *et al.*, 2004; Zhang *et al.*, 2006; Weijers *et al.*, 2007).

Lipid analysis identified crenarchaeol as the major GDGT component of the HL72 culture (Fig. 5). As this could not originate from inoculum carry-over following over 100 10% serial transfers of the culture, crenarchaeol must derive from archaeal synthesis at 72°C. The ratio of crenarchaeol (I) to GDGT-II in HL72 (I/I + II = 0.9) is well within the distribution versus temperature observed by Zhang and colleagues (2006) for other hot spring systems and is similar to that reported for *C. symbiosum* (I/I + II = 0.7, calculated from Sinninghe Damsté *et al.*, 2002a,b). Non-isoprenoid tetraether lipids were not detected in HL72. However, in addition to dialkyl tetraether lipids (GDGTs I-VII), we also identified a series of trialkyl-type tetraether lipids in both enrichment cultures and in hot spring samples (Fig. 5). The most abundant of these trialkyl lipids was compound VIII (Fig. S3), which

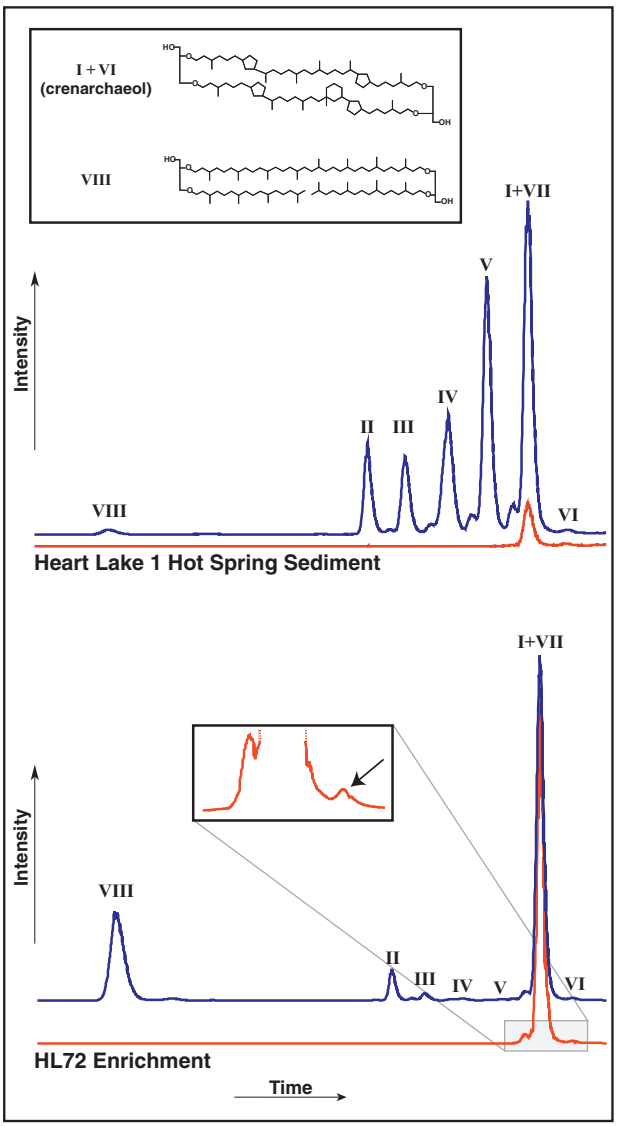


Fig. 5. Archaeal isoprenoid ether membrane lipids of Heart Lake 1 sediment and HL72 culture. HPLC-MS base peak chromatogram (blue line) and the extracted ion chromatogram for m/z 1292 (crenarchaeol and GDGT-VI) (red line) of lipids extracted from Heart Lake 1 sediment and HL72. Top inset shows the structures of crenarchaeol and its regioisomer (GDGTs I and VI) and the trialkyl-type caldarchaeol (VIII). Arrow in bottom inset indicates the extracted ion peak corresponding to the crenarchaeol regioisomer (GDGT-VI). Roman numerals above peaks correspond to GDGT structures in Fig. S3.

was also detected in extracts from Heart Lake 1 sediment (Fig. 5). Compound VIII has been reported as a minor constituent of the total ether lipid pool in a number of cultivated hyperthermophilic *Crenarchaeota* (Gulik *et al*., 1988; Hopmans *et al*., 2000; Uda *et al*., 2000) and has been proposed as an intermediate in the biosynthesis of GDGT-II from archaeol (GDGT-Ø) (Koga and Morii, 2007). Furthermore, traces of VIII are routinely found in non-thermophilic environments (A.E. Ingalls, unpubl. data). Hopmans and colleagues (2000) observed small amounts of VIII in marine sediments. Interestingly, the proportion of crenarchaeol to VIII varied with culture conditions, with higher abundances of VIII observed under suboptimal growth conditions (e.g. growth at 72°C at pH 6.0) (Fig. S4). Under these conditions, HL72 contains not only VIII, but also IX-XII (Fig. S4), presumably the trialkyl versions of GDGTs III-V (Fig. 5). The presence of these compounds in such high abundances may be an indication of physiological stress in the HL72 archaeon. The structures of compounds IX-XII have not been fully elucidated and may contain double bonds rather than rings. Nevertheless, to our knowledge, trialkyl tetraethers with more than one cyclopentyl ring (X-XII) have not been previously reported in either cultures or environmental samples.

Based on the data presented here, we propose the following provisional taxonomic assignment for the archaeon in the HL72 culture.

Nitrosocaldales order nov.
Nitrosocaldaceae fam. nov.
'*Candidatus* Nitrosocaldus yellowstonii'
General nov. et sp. nov.

Etymology. *Nitrosus* (Latin masculine adjective): nitrous; *caldus* (Latin masculine adjective): hot; *yellowstonii* (Latin masculine genitive name): from YNP. The name alludes to the physiology of the organism (ammonia oxidizer, thermophilic) and the habitat from which it was recovered.
Locality. The sediment from a terrestrial hot spring in YNP, USA.
Diagnosis. A thermophilic chemolithoautotrophic ammonia oxidizer from the domain *Archaea* appearing as small cocci or very short rods.

The discovery of an early diverging lineage of thermophilic ammonia-oxidizing *Crenarchaeota* significantly extends the known habitat range of nitrifying microorganisms and supports inferences based on gene surveys that the capacity for ammonia oxidation is broadly distributed among *Crenarchaeota*. Our observation of crenarchaeol biosynthesis by this thermophile is consistent with *in situ* production of this lipid in geothermal environments (Pearson *et al*., 2004; Zhang *et al*., 2006; Schouten *et al*., 2007). Although this is not incompatible with the suggestion that crenarchaeol is associated with the expansion of *Crenarchaeota* into moderate temperature habitats (Kuypers *et al*., 2001; Sinninghe Damsté *et al*., 2002a; Schouten *et al*., 2003), it may serve functions other than modifying membrane fluidity. For example, if this unique core lipid is a component of an archaeal cell-membrane associated system of ammonia oxidation, then it could be diagnostic primarily for ammonia oxidation. These findings also raise the possibilities that ammonia oxidation originated within thermophilic *Archaea* and that a complete nitrogen cycle may function at much higher temperatures than now appreciated.

Experimental procedures

Characterization of sampling sites

Sediment samples (~150 g of sediment and ~50 ml of spring water) were collected from a variety of hydrothermal features in YNP in previously sterilized Pyrex glass bottles and homogenized in the field by stirring with a sterile pipette. Approximately 50 ml of this sediment slurry was transferred to a sterile conical tube, frozen on dry ice within 2h of collection and kept at –80°C until processed for molecular or lipid analyses. The remaining sample, destined for cultivation studies, was transported back to the laboratory at room temperature and stored at 4°C. Temperature, pH and salinity were measured *in situ* using both a combined temperature-pH probe (pH 330; WTW, Fort Myers, FL) and a YSI30 portable instrument (YSI, Yellow Springs, OH). Concentrations of inorganic nitrogen compounds were determined on site, in triplicate, using a DR/820 portable colorimeter and standard test kits (Hach, Loveland, CO).

Cultivation of a thermophilic AOA

Enrichment cultures were incubated aerobically, in the dark and without shaking, at 60–80°C in Synthetic Freshwater *Crenarchaeota* Medium containing 1 g l^{-1} NaCl, 0.4 g l^{-1} $MgCl_26H_2O$, 0.1 g l^{-1} $CaCl_22H_2O$ and 0.5 g l^{-1} KCl. All other components are as previously described (Könneke *et al*., 2005). Medium was aliquoted into Balch tubes (10 ml per tube), the headspace (~25 ml) exchanged with N_2/CO_2 (80%/20%) and the tubes sealed with butyl-rubber stoppers. Prior to inoculation, 2.5 ml of 100% O_2 was added to the headspace. Sediment slurries (0.5 g) were used as inoculum. Growth was monitored by microscopy, nitrite production and fluorescent *in situ* hybridization (Könneke *et al*., 2005). Cultures were routinely transferred (10% volume per transfer) into fresh medium when nitrite concentrations approached 0.9 mM, indicating consumption of ~90% of the available ammonium.

Molecular analysis of 16S rRNA and putative archaeal amo *genes*

DNA for molecular analyses of environmental samples was extracted from 0.5 g of hot spring sediment using the Fast DNA kit for Soil (Qbiogene, Carlsbad, CA) according to the manufacturer's instructions. For cultures, cells were har-

vested by filtration onto 0.2 μm polycarbonate filters after first vortexing culture tubes to collect material that might be attached to the tube walls (no such biofilms were observed in any culture). Genomic DNA was then extracted directly from the filter as described above for sediment samples. Near-complete 16S rRNA genes were PCR amplified using either bacterial- [27F and 1492R (Lane, 1991)] or archaeal-specific primers [Arch21F and either Arch958R (DeLong, 1992)] or ArchLSU51R [García-Martínez and Rodríguez-Valera, (2000)] with the following protocol: 94°C for 4 min; 30 cycles consisting of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s; and 72°C for 10 min. Putative archaeal ammonia monooxygenase A subunit genes were amplified using a reverse primer for the *amoA* gene [Arch–amoAR (Francis *et al*., 2005)] and a forward primer based on *amoA*-like sequences in public databases (Arch_amoA_F 5′-AATGGTCTGGS TTAGAMG-3′) using the protocol described above for rRNA genes but with an annealing temperature of 52°C. Amplifications of genome fragments containing genes encoding more than one AMO subunit were carried out using Arch–amoAR and a reverse primer for the putative amoB gene [CrenAmo2.2R (Könneke *et al*., (2005)] as described above for the amplification of *amoA* genes but with an extension time of 2 min at 72°C. Amplification of bacterial *amoA* genes was examined using existing primer sets and reaction conditions (Rotthauwe *et al*., 1997). Plasmids containing copies of the 16S rRNA and *amoA*-like genes of *N. maritimus* (Könneke *et al*., 2005) and the *amoA* gene of *Nitrosomonas europaea* were used to optimize PCR conditions. Amplified fragments were cloned using a TOPO-TA Cloning Kit (Invitrogen) and sequenced as previously described (Könneke *et al*., 2005). Phylogenetic analyses of the archaeal rRNA and *amoA*-like sequences were carried out using evolutionary distance, parsimony and maximum likelihood methods using the ARB (Ludwig *et al*., 2004) and Phylip (Felsenstein, 2005) software packages.

Fluorescence in situ *hybridization*

For FISH analysis, cultures were first mixed by vortexing, then cells were fixed in 2% paraformaldehyde and filtered onto 0.2 mm polycarbonate GTBP membranes (Millipore). Cy3-labelled archaeal (Arc915) and FITC-labelled bacterial (Eub338-I, II and III) specific probes were used as previously described (Amann *et al*., 1990).

Extraction and analysis of archaeal membrane lipids

Centrifuged or filtered (0.2 μm) culture medium, and freeze-dried hot spring samples were extracted using a modified Bligh and Dyer protocol in which samples were ultrasonically extracted with 2:1:0.8 v/v methanol/dichloromethane/50 mM trichloroacetate (Nishihara and Koga, 1987; Koga and Morii, 2007). Crude extracts were further extracted with dichloromethane (three times), washed with deionized water and dried under a stream of N_2. The dried total extracts were dissolved in hexane/isopropanol (99:1) and analysed by liquid chromatography mass spectrometry (LC-MS) without further treatment (Hopmans *et al*., 2000; 2004). Analyses were carried out on an Agilent (Palo Alto, CA, USA) 1100 series LC fitted with a Prevail Cyano column (2.1 × 150 mm, 3 μm; Alltech, Deerfield, IL, USA). Lipids were detected by atmospheric pressure positive ion chemical ionization mass spectrometry on an Agilent ion trap MS with the following specifications: nebulizer pressure: 60 psi, drying gas pressure: 7 psi, scanning 1250–1350 m/z.

Accession numbers

The sequences described in this manuscript have been deposited in GenBank under accession numbers EU239959–EU240001.

Acknowledgements

We thank Allison R. Blevins, Nicolás Pinel, Willm Martens-Habbena and Paul Berube for technical assistance, invaluable discussions and help in the field, Ann Pearson for helpful comments on the manuscript and the National Park Service, particularly Christie Hendrix and Christine Smith, for permission to perform research in Yellowstone National Park. This work was supported by NSF Systematics grant DEB-0213186 (D.A.S.), NSF Microbial Interactions and Processes grant MCB-0604448 (to D.A.S. and J.R.T) and NSF Biological Oceanography grant OCE-0623174 (to A.E.I. and D.A.S.).

References

Amann, R.I., Krumholz, L., and Stahl, D.A. (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J Bacteriol* **172:** 762–770.

Church, M.J., DeLong, E.F., Ducklow, H.W., Karner, M.B., Preston, C.M., and Karl, D.M. (2003) Abundance and distribution of planktonic Archaea and Bacteria in the waters west of the Antarctic Peninsula. *Limnol Oceanogr* **48:** 1893–1902.

Coolen, M.J., Abbas, B., van Bleijswijk, J., Hopmans, E.C., Kuypers, M.M., Wakeham, S.G., and Sinninghe Damsté, J.S. (2007) Putative ammonia-oxidizing Crenarchaeota in suboxic waters of the Black Sea: a basin-wide ecological study using 16S ribosomal and functional genes and membrane lipids. *Environ Microbiol* **9:** 1001–1016.

DeLong, E.F. (1992) *Archaea* in coastal marine environments. *Proc Natl Acad Sci USA* **89:** 5685–5689.

Felsenstein, J. (2005) PHYLIP (Phylogeny Inference Package). Distributed by Author. http://evolution.genetics.washington.edu/phylip.html

Francis, C.A., Roberts, K.J., Beman, J.M., Santoro, A.E., and Oakley, B.B. (2005) Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proc Natl Acad Sci USA* **102:** 14683–14688.

García-Martínez, J., and Rodríguez-Valera, F. (2000) Microdiversity of uncultured marine prokaryotes: the SAR11 cluster and the marine Archaea of Group I. *Mol Ecol* **9:** 935–948.

Griffin, B.M., Schott, J., and Schink, B. (2007) Nitrite, an electron donor for anoxygenic photosynthesis. *Science* **316:** 1870.

Gulik, A., Luzzati, V., DeRosa, M., and Gambacorta, A. (1988) Tetraether lipid components from a thermoacidophilic archaebacterium. Chemical structure and physical polymorphism. *J Mol Biol* **201:** 429–435.

Hallam, S.J., Mincer, T.J., Schleper, C., Preston, C.M., Roberts, K., Richardson, P.M., and Delong, E.F. (2006) Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analyses of marine crenarchaeota. *PLoS Biol* **4:** e95.

Hirayama, H., Takai, K., Inagaki, F., Yamato, Y., Suzuki, M., Nealson, K.H., and Horikoshi, K. (2005) Bacterial community shift along a subsurface geothermal water stream in a Japanese gold mine. *Extremophiles* **9:** 169–184.

Hopmans, E.C., Schouten, S., Pancost, R.D., van der Meer, M.T., and Sinninghe Damsté, J.S. (2000) Analysis of intact tetraether lipids in archaeal cell material and sediments by high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. *Rapid Commun Mass Spectrom* **14:** 585–589.

Hopmans, E.C., Weijers, J.W.H., Schefuß, E., Herfort, L., Sinninghe Damsté, J.S., and Schouten, S. (2004) A novel proxy for terrestrial organic matter in sediments based on branched and isoprenoid tetraether lipids. *Earth Planet Sci Lett* **224:** 107–116.

Karner, M.B., DeLong, E.F., and Karl, D.M. (2001) Archaeal dominance in the mesopelagic zone of the Pacific Ocean. *Nature* **409:** 507–510.

Koga, Y., and Morii, H. (2007) Biosynthesis of ether-type polar lipids in archaea and evolutionary considerations. *Microbiol Mol Biol Rev* **71:** 97–120.

Könneke, M., Bernhard, A.E., de la Torre, J.R., Walker, C.B., Waterbury, J.B., and Stahl, D.A. (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* **437:** 543–546.

Kuypers, M.M., Blokker, P., Erbacher, J., Kinkel, H., Pancost, R.D., Schouten, S., and Sinninghe Damsté, J.S. (2001) Massive expansion of marine archaea during a mid-Cretaceous oceanic anoxic event. *Science* **293:** 92–95.

Lam, P., Jensen, M.M., Lavik, G., McGinnis, D.F., Muller, B., Schubert, C.J., *et al.* (2007) Linking crenarchaeal and bacterial nitrification to anammox in the Black Sea. *Proc Natl Acad Sci USA* **104:** 7104–7109.

Lane, D.J. (1991) 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*. Stackebrandt, E., and Goodfellow, M. (eds). New York, NY, USA: John Wiley and Sons, pp. 115–175.

Lebedeva, E.V., Alawi, M., Fiencke, C., Namsaraev, B., Bock, E., and Spieck, E. (2005) Moderately thermophilic nitrifying bacteria from a hot spring of the Baikal rift zone. *FEMS Microbiol Ecol* **54:** 297–306.

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G.W., *et al.* (2006) Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature* **442:** 806–809.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32:** 1363–1371.

Marteinsson, V.T., Hauksdottir, S., Hobel, C.F., Kristmannsdottir, H., Hreggvidsson, G.O., and Kristjansson, J.K. (2001) Phylogenetic diversity analysis of subterranean hot springs in Iceland. *Appl Environ Microbiol* **67:** 4242–4248.

Mehta, M.P., and Baross, J.A. (2006) Nitrogen fixation at 92 degrees C by a hydrothermal vent archaeon. *Science* **314:** 1783–1786.

Nicol, G.W., and Schleper, C. (2006) Ammonia-oxidising Crenarchaeota: important players in the nitrogen cycle? *Trends Microbiol* **14:** 207–212.

Nishihara, M., and Koga, Y. (1987) Extraction and composition of polar lipids from the archaebacterium, Methanobacterium thermoautotrophicum: effective extraction of tetraether lipids by an acidified solvent. *J Biochem (Tokyo)* **101:** 997–1005.

Nunoura, T., Hirayama, H., Takami, H., Oida, H., Nishi, S., Shimamura, S., *et al.* (2005) Genetic and functional properties of uncultivated thermophilic crenarchaeotes from a subsurface gold mine as revealed by analysis of genome fragments. *Environ Microbiol* **7:** 1967–1984.

Pearson, A., Huang, Z., Ingalls, A.E., Romanek, C.S., Wiegel, J., Freeman, K.H., *et al.* (2004) Nonmarine crenarchaeol in Nevada hot springs. *Appl Environ Microbiol* **70:** 5229–5237.

Risgaard-Petersen, N., Langezaal, A.M., Ingvardsen, S., Schmid, M.C., Jetten, M.S., Op den Camp, H.J., *et al.* (2006) Evidence for complete denitrification in a benthic foraminifer. *Nature* **443:** 93–96.

Rotthauwe, J.H., Witzel, K.P., and Liesack, W. (1997) The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl Environ Microbiol* **63:** 4704–4712.

Schouten, S., Hopmans, E.C., Pancost, R.D., and Sinninghe Damsté, J.S. (2000) Widespread occurrence of structurally diverse tetraether membrane lipids: evidence for the ubiquitous presence of low-temperature relatives of hyperthermophiles. *Proc Natl Acad Sci USA* **97:** 14421–14426.

Schouten, S., Hopmans, E.C., Forster, A., van Breugel, Y., Kuypers, M.M.M., and Sinninghe Damsté, J.S. (2003) Extremely high sea-surface temperatures at low latitudes during the middle Cretaceous as revealed by archaeal membrane lipids. *Geology* **31:** 1069–1072.

Schouten, S., van der Meer, M.T., Hopmans, E.C., Rijpstra, W.I., Reysenbach, A.L., Ward, D.M., and Sinninghe Damsté, J.S. (2007) Archaeal and bacterial glycerol dialkyl glycerol tetraether lipids in hot springs of Yellowstone National Park (USA). *Appl Environ Microbiol* **73:** 6181–6191.

Sinninghe Damsté, J.S., Schouten, S., Hopmans, E.C., van Duin, A.C., and Geenevasen, J.A. (2002a) Crenarchaeol: the characteristic core glycerol dibiphytanyl glycerol tetraether membrane lipid of cosmopolitan pelagic crenarchaeota. *J Lipid Res* **43:** 1641–1651.

Sinninghe Damsté, J.S., Rijpstra, W.I., Hopmans, E.C., Prahl, F.G., Wakeham, S.G., and Schouten, S. (2002b) Distribution of membrane lipids of planktonic *Crenarchaeota* in the Arabian Sea. *Appl Environ Microbiol* **68:** 2997–3002.

Spear, J.R., Barton, H.A., Robertson, C.E., Francis, C.A., and Pace, N.R. (2007) Microbial Community Biofabrics in a Geothermal Mine Adit. *Appl Environ Microbiol* **73:** 6172–6180.

Strous, M., Fuerst, J.A., Kramer, E.H., Logemann, S., Muyzer, G., van de Pas-Schoonen, K.T., *et al.* (1999) Missing lithotroph identified as new planctomycete. *Nature* **400:** 446–449.

Takai, K., Moser, D.P., DeFlaun, M., Onstott, T.C., and Fredrickson, J.K. (2001) Archaeal diversity in waters from deep South African gold mines. *Appl Environ Microbiol* **67:** 5750–5760.

Treusch, A.H., Leininger, S., Kletzin, A., Schuster, S.C., Klenk, H.-P., and Schleper, C. (2005) Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environ Microbiol* **7:** 1985–1995.

Uda, I., Sugai, A., Itoh, Y.H., and Itoh, T. (2000) Characterization of caldarchaetidylglycerol analogs, dialkyl-type and trialkyl-type, from Thermoplasma acidophilum. *Lipids* **35:** 1155–1157.

Weidler, G.W., Dornmayr-Pfaffenhuemer, M., Gerbl, F.W., Heinen, W., and Stan-Lotter, H. (2007) Communities of archaea and bacteria in a subsurface radioactive thermal spring in the Austrian Central Alps, and evidence of ammonia-oxidizing Crenarchaeota. *Appl Environ Microbiol* **73:** 259–270.

Weijers, J.W.H., Schouten, S., van den Donker, J.C., Hopmans, E.C., and Sinninghe Damsté, J.S. (2007) Environmental controls on bacterial tetraether membrane lipid distribution in soils. *Geochim Cosmochim Acta* **71:** 703–713.

Wuchter, C., Abbas, B., Coolen, M.J., Herfort, L., van Bleijswijk, J., Timmers, P., *et al.* (2006) Archaeal nitrification in the ocean. *Proc Natl Acad Sci USA* **103:** 12317–12322.

Zhang, C.L., Pearson, A., Li, Y.L., Mills, G., and Wiegel, J. (2006) Thermophilic temperature optimum for crenarchaeol synthesis and its implication for archaeal evolution. *Appl Environ Microbiol* **72:** 4419–4422.

Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Temperature dependence of nitrite production in HL72. Triplicate cultures of HL72 were incubated at the appropriate temperatures, nitrite concentrations were measured every 6–12 h and the maximal nitrite production rate calculated for each replicate. Values in the graph represent the mean of the maximal nitrite production rates calculated for each replicate. Errors bars indicate one standard deviation of the mean.

Fig. S2. Schematic representation of the genomic organization of *amo* genes in '*Candidatus* Nitrosocaldus yellowstonii' compared with *N. maritimus*, *C. symbiosum* and environmental genome fragments from marine and soil *Crenarchaeota*.

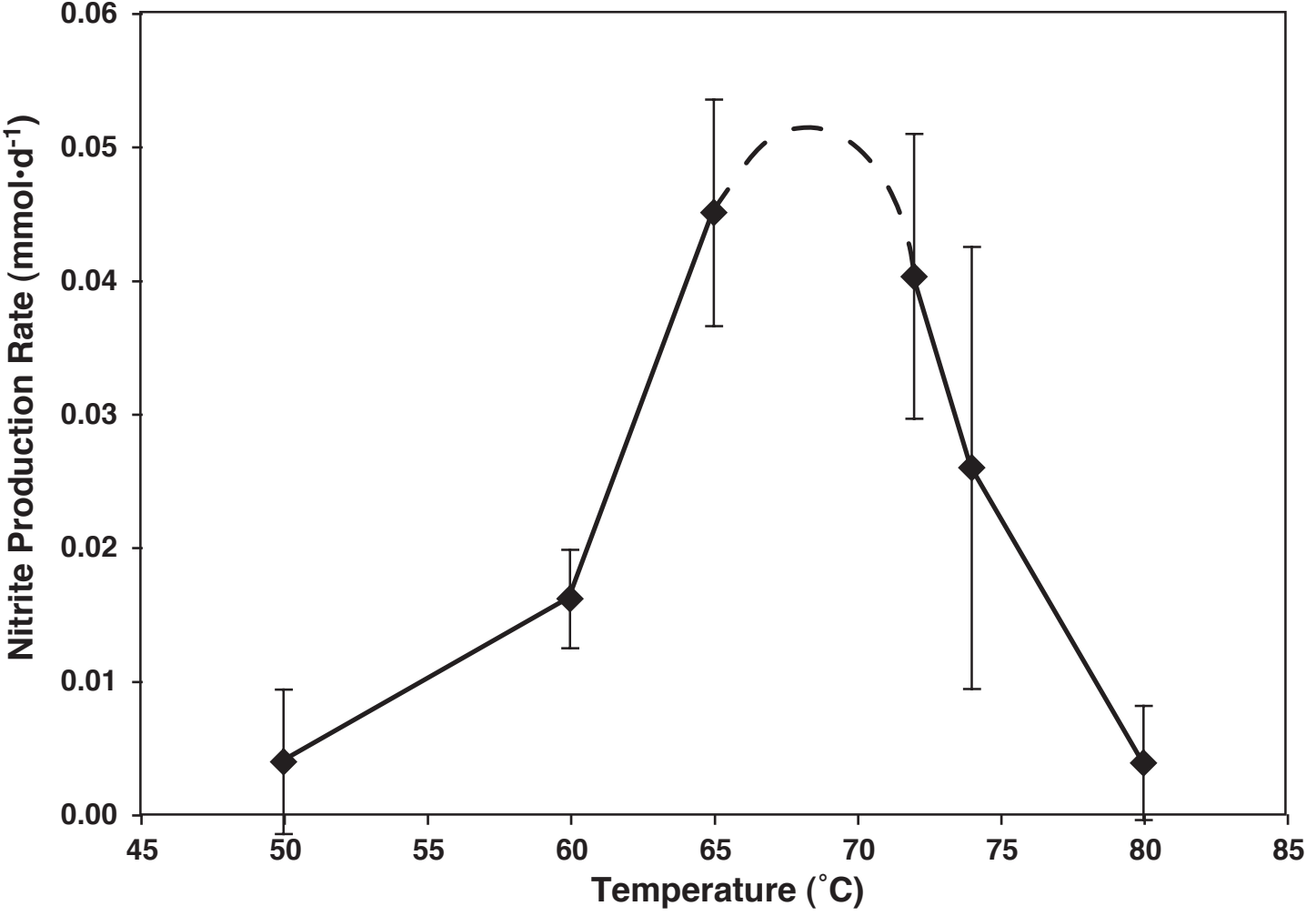
Fig. S3. Structures of archaeal GDGT lipids present in HL72 and in sediments from Yellowstone National Park. Structures of archaeol (Ø; two molecules shown – one in black, another in grey), GDGT-II-IV, crenarchaeol (I and isomer VI) and compound VIII (trialkyl-type GDGT-II).

Fig. S4. Archaeal isoprenoid ether membrane lipids of HL72 grown under suboptimal conditions (72°C, pH 6.0). HPLC-MS base peak chromatogram of lipids extracted from a culture of HL72 grown at 72°C and pH 6.0. Roman numerals above peaks correspond to GDGT structures in Fig. S3. Note the higher relative abundance of trialkyl tetraether lipids (compounds VIII–XII) compared with the chromatogram in Fig. 5. The structure of VIII (trialkyl-type GDGT-II) was verified by MS/MS analysis. The molecular ion of VIII has an m/z of 1304 and fragmentation resulted in ions with m/z of 1024, 1006, 988, 950 and 932 representing a loss of one phytanyl group as phytene and subsequent losses of one or two glycerol moieties and water respectively.

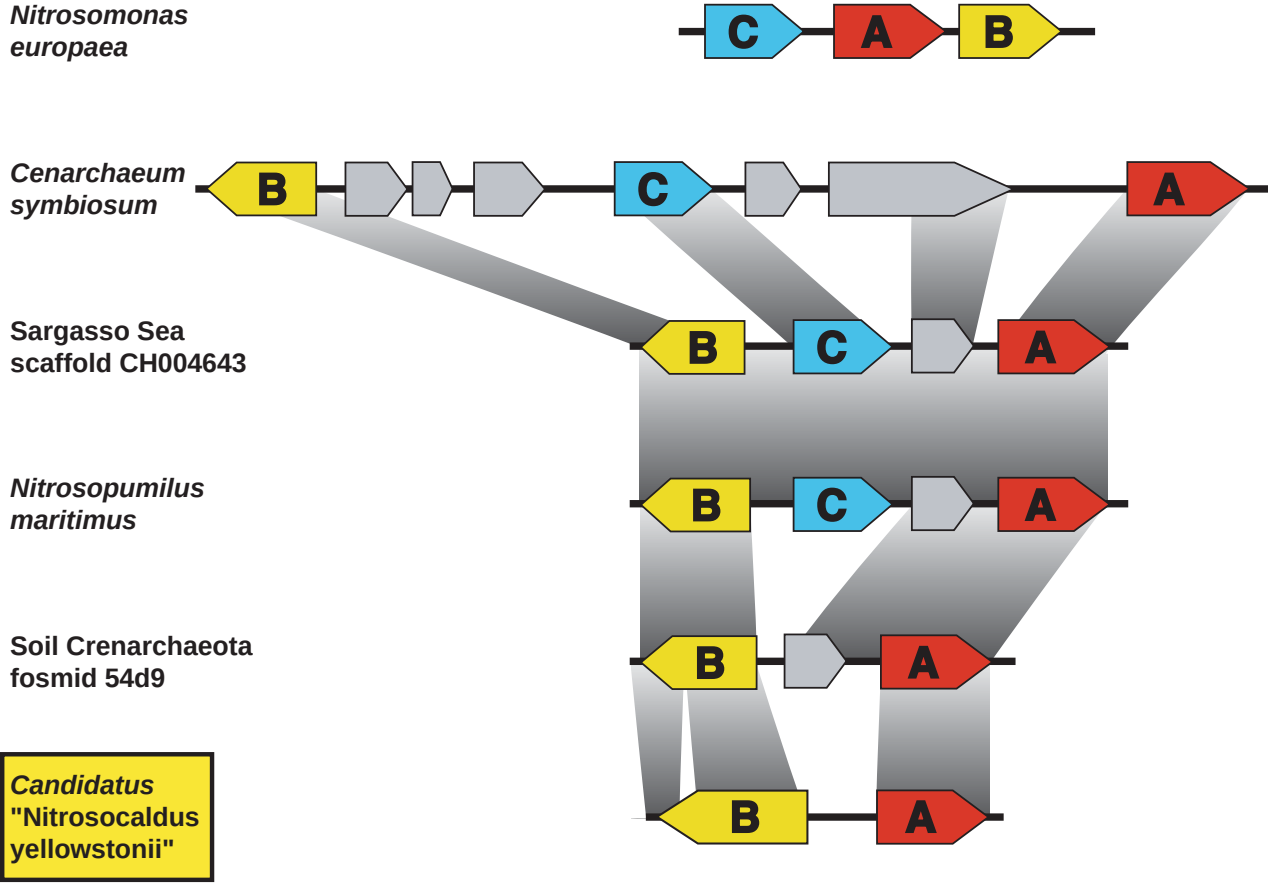
Table S1. Physical and chemical properties of YNP sampling sites used for the detection of thermophilic AOA.

This material is available as part of the online article from http://www.blackwell-synergy.com

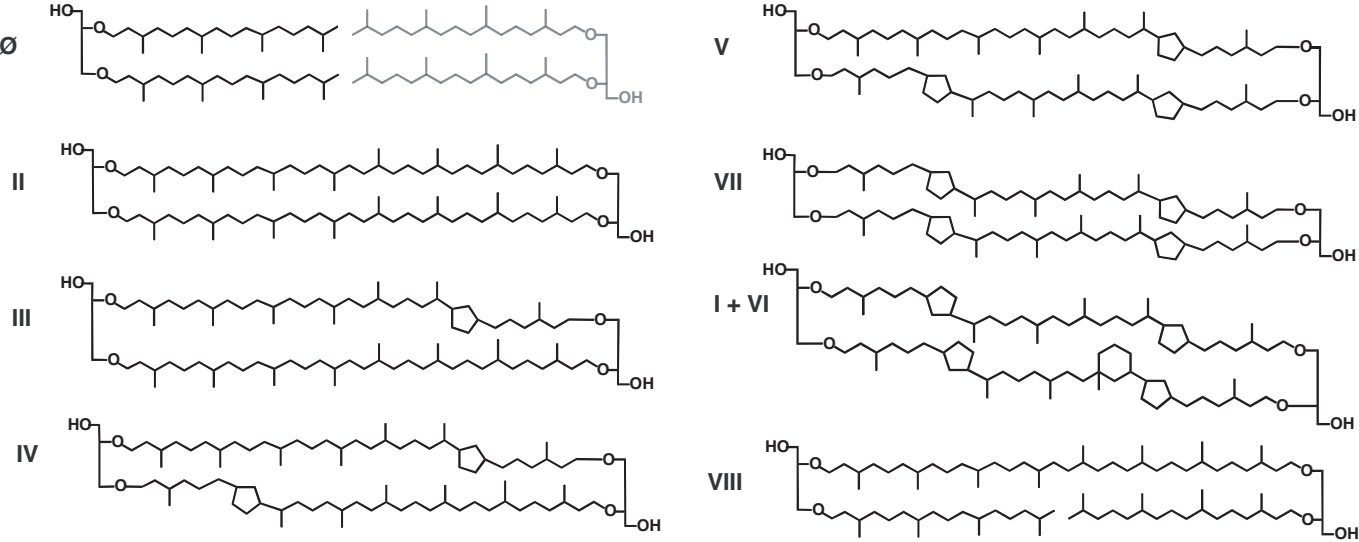
Please note: Blackwell Publishing are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.



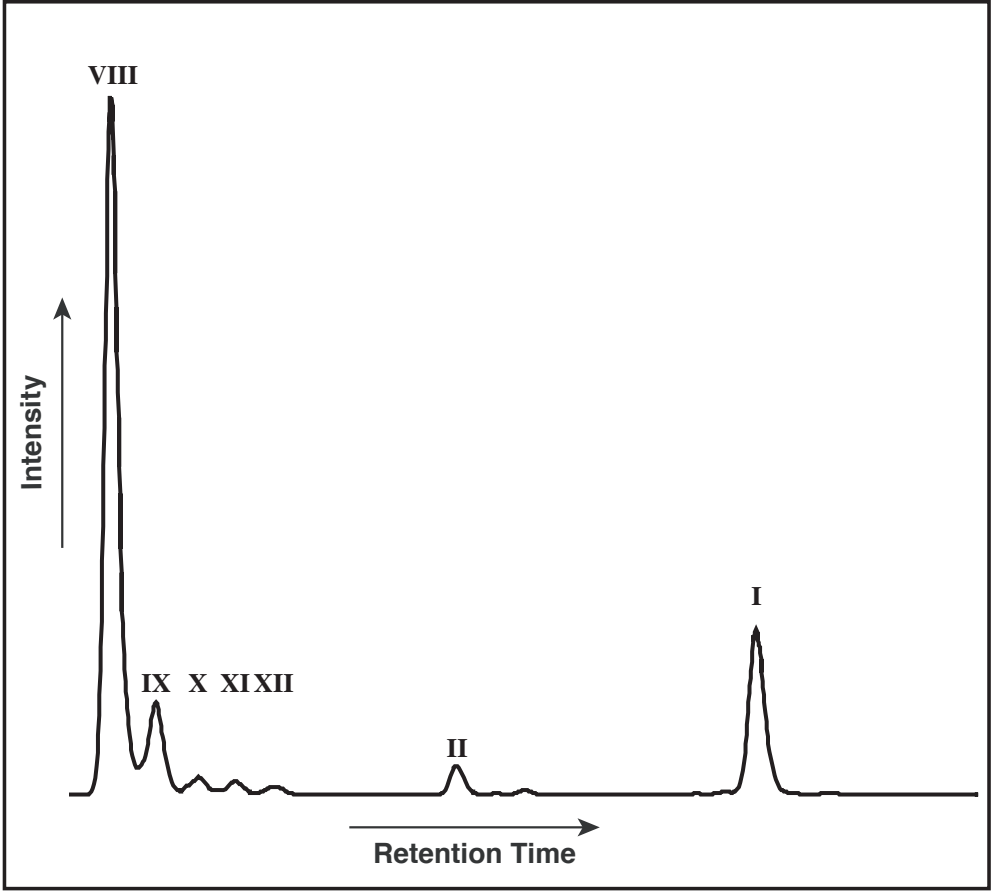
Supplementary Figure S1: Temperature-dependence of nitrite production in HL72



Supplementary Figure S2: Schematic representation of the genomic organization of *amo*-like genes in AOA



Supplementary Figure S3: Structures of archaeal GDGT lipids present in HL72 and in sediments from Yellowstone National Park



Supplementary Figure S4: HL72 lipids under suboptimal growth conditions (72˚C, pH 6.0)

Table S1. Physical and chemical properties of YNP sampling sites used for the detection of thermophilic AOA

Site	Abbr [a]	Sample Type	Temp. (˚C)	pH	Longitude	Latitude
Heart Lake 1	HL1	sediment	70-80	8.30	44˚18.139' N	110˚31.196' W
Heart Lake 3	HL3	sediment	78.9	7.22	44˚17.894' N	110˚31.051' W
Heart Lake 4	HL4	sediment	76.8	8.01	44˚17.886' N	110˚31.044' W
Heart Lake 5	HL5	sediment	82.5-90	6.90	44˚18.312' N	110˚31.326' W
Witch's Navel	WN	sediment	69.8	7.62	44˚18.255' N	110˚31.277' W
Octopus Spring	OS	sediment	70-90	8.02	44˚32.043' N	110˚47.870' W
O1A	O1a	microbial mat	72	*n/d*	44˚31.933' N	110˚47.800' W
Clearwater Spring	CS	sediment	54	6.2	*n/a*	*n/a*
Mammoth Spring	MamSp	sediment	*n/a*	*n/d*	*n/a*	*n/a*
Nymph Creek	NyCr	microbial mat	60	3.00	44˚45.176' N	110˚43.447' W
Shoshone Spring 7	SHO7	sediment	70	8.0-9.0	44˚21.190' N	110˚48.076' W
Obsidian Pool fumarole	OPF	microbial mat	95	*n/d*	44˚36.605' N	110˚26.331' W

[a] *Abbreviations used for clone names in Figures 1 and 3*
n/a, not available; n/d, data not determined