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Article in *International Journal of Systematic and Evolutionary Microbiology* · October 2017

DOI: 10.1099/ijsem.0.002416

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Nitrosopumilus maritimus gen. nov., sp. nov., *Nitrosopumilus cobalaminigenes* sp. nov., *Nitrosopumilus oxycliniae* sp. nov., and *Nitrosopumilus ureiphilus* sp. nov., four marine ammonia-oxidizing archaea of the phylum *Thaumarchaeota*

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Abstract

Four mesophilic, neutrophilic, and aerobic marine ammonia-oxidizing archaea, designated strains SCM1^T, HCA1^T, HCE1^T and PSO^T, were isolated from a tropical marine fish tank, dimly lit deep coastal waters, the lower euphotic zone of coastal waters, and near-surface sediment in the Puget Sound estuary, respectively. Cells are straight or slightly curved small rods, 0.15–0.26 µm in diameter and 0.50–1.59 µm in length. Motility was not observed, although strain PSO^T possesses genes associated with archaeal flagella and chemotaxis, suggesting it may be motile under some conditions. Cell membranes consist of glycerol dibiphytanyl glycerol tetraether (GDGT) lipids, with crenarchaeol as the major component. Strain SCM1^T displays a single surface layer (S-layer) with p6 symmetry, distinct from the p3-S-layer reported for the soil ammonia-oxidizing archaeon *Nitrososphaera viennensis* EN76^T. Respiratory quinones consist of fully saturated and monounsaturated menaquinones with 6 isoprenoid units in the side chain. Cells obtain energy from ammonia oxidation and use carbon dioxide as carbon source; addition of an α -keto acid (α -ketoglutaric acid) was necessary to sustain growth of strains HCA1^T, HCE1^T, and PSO^T. Strain PSO^T uses urea as a source of ammonia for energy production and growth. All strains synthesize vitamin B₁ (thiamine), B₂ (riboflavin), B₆ (pyridoxine), and B₁₂ (cobalamin). Optimal growth occurs between 25 and 32 °C, between pH 6.8 and 7.3, and between 25 and 37 ‰ salinity. All strains have a low mol% G+C content of 33.0–34.2. Strains are related by 98 % or greater 16S rRNA gene sequence identity, sharing ~85 % 16S rRNA gene sequence identity with *Nitrososphaera viennensis* EN76^T. All four isolates are well separated by phenotypic and genotypic characteristics and are here assigned to distinct species within the genus *Nitrosopumilus* gen. nov. Isolates SCM1^T (=ATCC TSD-97^T =NCIMB 15022^T), HCA1^T (=ATCC TSD-96^T), HCE1^T (=ATCC TSD-98^T), and PSO^T (=ATCC TSD-99^T) are type strains of the species *Nitrosopumilus maritimus* sp. nov., *Nitrosopumilus cobalaminigenes* sp. nov., *Nitrosopumilus oxycliniae* sp. nov., and *Nitrosopumilus ureiphilus* sp. nov., respectively. In addition, we propose the family *Nitrosopumilaceae* fam. nov. and the order *Nitrosopumilales* ord. nov. within the class *Nitrososphaeria*.

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Keywords: *Thaumarchaeota*; nitrification; *Nitrosopumilus*; *Nitrosopumilaceae*; *Nitrosopumilales*; Vitamin B₁₂.

Abbreviations: Ado-Cbl, adenosylcobalamin; AMO, ammonia monooxygenase; *amoA*, the gene coding for the α -subunit of the AMO; ANI, average nucleotide identity; AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria; CO₂, carbon dioxide; cren', crenarchaeol regioisomer; Cu-MMO, copper-containing membranebound monooxygenase; ECT, Electron CryoTomography; *ectABCD*, ectoine/hydroxyectoine biosynthetic genes; GDGT, glycerol dibiphytanyl glycerol tetraether; HP/HB, 3-hydroxypropionate/4-hydroxybutyrate; *K_m*, half saturation constant; LC-MS, liquid-chromatography mass spectrometry; Me-Cbl, methylcobalamin; MGI, mesophilic marine Group I; NO, nitric oxide; N₂O, nitrous oxide; OH-Cbl, hydroxycobalamin; O₂, oxygen; PRISM, Puget Sound Regional Synthesis Model; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; SCM, Synthetic Crenarchaeota Medium; S-layer, surface layer; TEM, transmission electron microscopy; ThAOA, thermophilic ammonia-oxidizing archaea.

Footnote: The names *Nitrosopumilus cobalaminigenes*, *Nitrosopumilus oxycliniae* and *Nitrosopumilus ureiphilus* are effectively published in this paper. Due to problems connected with the deposition of the type strains of the proposed species, valid publication of these names is currently not possible. Eight supplementary figures are available with the online Supplementary Material.

INTRODUCTION

For decades, *Archaea* were defined as obligate extremophiles and restricted to high-temperature, extremely acidic, hypersaline, or strictly anoxic habitats [1]. However, this perception was overturned with the discovery of mesophilic marine Group I (MGI) and Group II *Archaea* from temperate and oxygenated ocean waters [2, 3]. Detailed molecular surveys indicated that MGI are among the most ubiquitous and abundant marine prokaryotes, approaching 40 % of total bacterioplankton in the meso- and bathypelagic zones [4], and constituting a considerable fraction of the microbial biomass in the ocean [5]. Marine metagenomic studies first hinted at the metabolic potential of MGI, as a copper-containing membrane-bound monooxygenase (Cu-MMO) gene was found on a MGI-associated scaffold [6]. Although Cu-MMOs consist of a diverse family of enzymes having broad substrate range, this gene was provisionally annotated as coding for an ammonia monooxygenase (AMO), based on ~25 % amino acid identity with bacterial AMO [6], although also sharing significant identity (~50 %) with the bacterial particulate methane monooxygenase [7, 8]. Therefore, demonstration of a capacity for archaeal ammonia oxidation awaited the isolation of the first chemolithoautotrophic marine ammonia-oxidizing archaeon (AOA) *Nitrosopumilus maritimus* SCM1^T, which established the definitive link between the MGI AMO-encoding genes and nitrification [9]. Subsequent extensive archaeal *amoA* (the gene coding for the α -subunit of the AMO) gene surveys demonstrated the widespread presence of the putative *amoA* genes in divergent MGI, suggestive of their significant role in marine nitrification [10]. However, given the extremely low ammonia concentrations in the ocean and the thermodynamically low energy yield of ammonia oxidation ($\Delta G^{\circ} = -271 \text{ kJ mol}^{-1} \text{ NH}_4^+$) [11], it remained uncertain whether all members of MGI obligatorily depend on ammonia as the sole energy source for growth. For example, Agogué *et al.* [12] speculated that most deep-sea MGI are incapable of autotrophic ammonia oxidation and are likely heterotrophs using organic matter for growth based on a marked decrease in the ratio of *amoA*: MGI 16S rRNA genes with depth [12]. This vertical gradient of decreasing ratio was however later shown to be mainly caused by primer bias [13]. Using strain SCM1^T as a model organism, detailed studies of ammonia oxidation kinetics and the biochemical characterizations of the carbon dioxide (CO₂) fixation pathway identified adaptive features associated with growth under extreme ammonia limitation [14, 15]. Strain SCM1^T has a remarkably low half saturation constant (K_m) for ammonia oxidation of 133 nM total ammonia (NH₃ + NH₄⁺) and an exceptionally high ammonia affinity of 68 700 l g⁻¹ cells h⁻¹, which is among the highest substrate affinities yet reported for any microorganism [14]. High-affinity for ammonia is coupled with the most energy-efficient aerobic pathway for carbon fixation yet characterized, which together are thought to contribute to the remarkable ecological success of oligotrophic

marine AOA [14, 15]. It is now widely accepted that they are major contributors of marine nitrification [16–18], and appear to be almost completely responsible for ammonia oxidation in oligotrophic oceans [19].

Since the isolation of the first representative strain SCM1^T, the same general cultivation strategy has been widely applied to culture additional members of archaeal ammonia oxidizers from a variety of habitats, including marine, soil, freshwater, wastewater, and hot springs [20]. Available AOA cultures grow at temperatures as high as 74 °C (*Candidatus Nitrosocaldus yellowstonensis* (formerly '*Candidatus Nitrosocaldus yellowstonii*') HL72) [21], pH as low as 4.0 (*Candidatus Nitrosotalea* sp. Nd2) [22], salinities between 55 and 96 ‰ (SCM1^T) [23, 24], oxygen (O₂) concentrations of 1 μM or lower (*Nitrosopumilus ureiphilus* PS0^T) [25, 26], and ammonia concentrations up to 100 mM (*Candidatus Nitrosocosmicus franklandus* C13) [27]. It has been shown that even phylogenetically closely related AOA strains display distinct physiological characteristics, supporting fine niche partitioning and ecological differentiation [22, 28, 29]. Despite the enormous metabolic and functional diversity, all sequenced AOA representatives share common genomic features such as a copper-based enzymatic system for ammonia oxidation and electron transfer, a variant of the autotrophic 3-hydroxypropionate/4-hydroxybutyrate (HP/HB) cycle for carbon fixation, and a complete cobalamin (vitamin B₁₂) synthesis pathway [30–35]. In addition, all investigated AOA strains synthesize the same type of membrane lipids, the glycerol dibiphytanyl glycerol tetraether (GDGT) lipids with crenarchaeol as the characteristic component [21, 23, 25, 36, 37], the apolar lipid methoxy archaeol [38], and the same suite of membrane-bound respiratory menaquinones with 6 isoprenoid units [39].

Archaeal ammonia oxidizers associate with the distinct and deeply-branching phylum *Thaumarchaeota*, which is divided into four major phylogenetic sub-lineages, group I.1a, group I.1a-associated, group I.1b, and ThAOA (Thermophilic AOA) [40, 41]. Members of marine AOA are almost exclusively affiliated with the group I.1a *Thaumarchaeota* [42]. Classification of AOA available in pure culture or enrichment embraces this general phylogenetic structure in consideration of additional ecological, physiological, and biochemical data [20, 43, 44]. However, few strains have been validly described. Here we report the formal taxonomic description of four closely related marine isolates: a novel marine thaumarchaeote from the primary nitrite maximum layer in Puget Sound coastal waters (strain HCE1^T) and the previously reported thaumarchaeotal isolates SCM1^T, HCA1^T and PS0^T. We extend the original descriptions of these three AOA isolates [28], presenting additional characterizations of the cell envelope structure and vitamin synthesis. Based on these data we formally propose the species *Nitrosopumilus maritimus* sp. nov., which is assigned as the type species of the genus *Nitrosopumilus* gen. nov. Furthermore, the physiological and genomic characteristics indicate that strains HCA1^T, HCE1^T and PS0^T

represent three novel species of this genus, for which the names *Nitrosopumilus cobalaminigenes* sp. nov., *Nitrosopumilus oxycliniae* sp. nov., and *Nitrosopumilus ureiphilus* sp. nov. are proposed, respectively. We also propose the genus *Nitrosopumilus* [45] as the type genus of the family *Nitrosopumilaceae* fam. nov. [46] and the order *Nitrosopumilales* ord. nov. [47] within the class *Nitrososphaeria* [48].

METHODS

Sample source and culture maintenance

Strain SCM1^T was isolated from a tropical marine fish tank at the Seattle Aquarium in Seattle, Washington, USA [9]. Strain HCA1^T was recovered from a depth of 50 m water at the Puget Sound Regional Synthesis Model (PRISM; www.prism.washington.edu) station P10 and strain HCE1^T at a depth of 17 m water (nitrite max) at the PRISM station P12, both in Hood Canal, Washington, USA [25, 28]. Strain PS0^T was obtained from a nearshore marine surface sediment in Seattle, Washington, USA (47.59 N, 122.40 W) [28]. The enrichment and isolation strategies were described previously by Könneke *et al.* [9] and Qin *et al.* [28]. Briefly, strains SCM1^T and PS0^T were enriched in bicarbonate-buffered Synthetic Crenarchaeota Medium (SCM) [9, 14, 28] supplemented with 1 mM and 500 μM NH₄Cl, respectively, while a lower amount of NH₄Cl (2 μM) was used to selectively enrich strains HCA1^T and HCE1^T. Growth was monitored by ammonium consumption, nitrite production and microscopic cell counts [9, 28]. Pure cultures were obtained via a combination of multiple isolation strategies, such as end-point dilution, antibiotic addition (40 mg l⁻¹ streptomycin), filtration (0.22- and 0.45 μm pore-size), and organic acid supplementation (100 μM α-ketoglutaric acid). Axenic strain SCM1^T was maintained in HEPES-buffered SCM containing (l⁻¹) 26 g NaCl, 5 g MgSO₄·7H₂O, 5 g MgCl₂·6H₂O, 1.5 g CaCl₂·2H₂O, 0.1 g KBr, 10 ml HEPES buffer (1 M HEPES and 0.6 M NaOH), 2 ml NaHCO₃ (1 M), 5 ml KH₂PO₄ (0.4 g l⁻¹), 1 ml FeNaEDTA solution (7.5 M), 1 ml trace element solution, and 1 ml NH₄Cl (1 M) [14]. Strains HCA1^T, HCE1^T and PS0^T were maintained in bicarbonate-buffered SCM containing 3 mM NaHCO₃ supplemented with 200 μM NH₄Cl, and 100 μM α-ketoglutaric acid [28]. The isolates were cultured in 2l flasks containing ~400 ml medium in the dark without shaking at 20 °C (HCA1^T, HCE1^T and PS0^T) or at 30 °C (SCM1^T).

Physiological characterization

We previously determined the growth response of strains SCM1^T, HCA1^T and PS0^T to varying temperature, pH, salinity, light, and O₂ concentration [25, 26, 28]. Similarly, in order to investigate the influence of key environmental variables on the growth of strain HCE1^T, late exponential or early stationary phase cultures were inoculated (1%, v/v transfer) into 100 ml HEPES-buffered SCM supplemented with 200 μM NH₄Cl and 100 μM α-ketoglutarate and incubated at temperatures ranging from 4 to 37 °C, at pH values ranging from 6.4 to 8.1, and at salinities ranging from 10 to 40‰. To assess the tolerance of all strains to high

concentrations of ammonia, cells from exponentially growing cultures were transferred to HEPES-buffered SCM with initial ammonia concentrations ranging from 200 μM to 50 mM NH₄Cl. No, or negligible, change in pH was observed between the beginning and end of each growth experiment.

The effect of organic substrates on the growth of strain SCM1^T was surveyed by inoculating an exponential phase culture (1% transfer) into 100 ml SCM individually supplemented with 64 different organic compounds (micromolar range). Three tricarboxylic acid cycle intermediates pyruvate, oxaloacetate, and α-ketoglutarate, which showed positive effects on the growth of SCM1^T, were selected as potential organic supplements for growth of strains HCA1^T, HCE1^T and PS0^T. These pure cultures were maintained in SCM supplemented with 100 μM α-ketoglutaric acid before investigating the effect of different organic substrates on their growth. Therefore, two consecutive transfers (1%, v/v) into organic-free medium were completed to minimize the carryover of α-ketoglutarate in the inoculum before addition of the other organic supplements. As previously reported [28], their lag phase was significantly extended and growth rate was decreased following the transfer into organic carbon-free medium. Growth in 100 ml SCM supplemented with 100 μM pyruvate, oxaloacetate, α-ketoglutarate, as well as glycolate, a common algal exudate, was compared to controls lacking organic carbon supplement. Growth was regularly monitored for two consecutive transfers (1%, v/v) in each medium. If not otherwise indicated, all growth experiments were carried out in triplicate in the dark without shaking.

Vitamins synthesis

To measure vitamin production from four AOA strains, cultures were grown at optimal growth temperature in 100–150 ml HEPES-buffered SCM without vitamin additions. Growth was monitored by nitrite production and microscopic cell counts as previously described by Qin *et al.* [28]. Strain SCM1^T was harvested at five time points on 0.22 μm Nylon membrane filters (Millipore), corresponding to the time of inoculation, mid-lag, early exponential, mid-exponential, and late exponential phases of growth. The other three strains were harvested in the late exponential phase on 0.22 μm Durapore membrane filters (Millipore). Cells were harvested in the dark to minimize vitamin photodegradation and stored at -20 °C prior to analysis. Cells were disrupted by bead beating in a solution of methanol: acetonitrile:water with formic acid (40:40:20 with 0.1% formic acid) [49] followed by analysis using liquid-chromatography mass spectrometry (LC-MS) [50], with modifications as previously described [35]. Vitamins B₁, B₂, and B₁₂ were measured as previously described [35, 50] and an active form of B₆ (pyridoxal phosphate) was monitored using a parent mass of 248.04 and daughter masses of 94.15 and 150.15 with collision energies of 28 and 16 V, respectively. For each vitamin compound, we relied on at least two

mass transitions matching the retention time of a standard to identify the compound.

Microscopy

Exponentially growing HCE1^T cells were prepared for transmission electron microscopy (TEM) examination as previously described by Qin *et al.* [28]. TEM images were recorded with a JEOL JEM-1400 transmission electron microscope operated at 120 kV and magnification of 15 000 and 20 000 \times (Electron Microscopy Core Facility, Fred Hutchinson Cancer Research Centre, Seattle, WA). Electron CryoTomography (ECT) was used to visualize the structural details of the surface layer (S-layer) of strain SCM1^T. Exponential phase cells of strain SCM1^T were concentrated by filtration and suspended in PBS containing BSA-treated colloidal gold fiducial markers (10 nm) [51, 52]. From this solution, 3 μ l was applied to R2/2 copper Quantifoil EM grids (Quantifoil Micro Tools). Using a vitrobot Mark III (FEI) maintained at 80 % humidity and 30 °C temperature, the excess liquid was blotted and the grids were plunged frozen into a liquid ethane-propane mixture. The cryopreserved grids were imaged using FEI PolaraTM G2 (FEI Company) 300 kV field emission gun transmission electron microscope. The tomograms (22 000 \times magnification) were collected and analysed using UCSF Tomo software (University of California, San Francisco) and IMOD software package.

Phylogenetic analysis

The 16S rRNA (1248 nucleic acid positions) and *amoA* (576 nucleic acid positions) sequences of currently available cultivated AOA representatives were first aligned using CLUSTALW and manually inspected [53]. The maximum-likelihood method with Kimura two-parameter correction and 500 bootstrap replicates was used to construct 16S rRNA and *amoA* gene phylogenetic trees using MEGA software version 7.0.14 [54]. The 16S rRNA and *amoA* sequences of strain SCM1^T were deposited previously in GenBank database (accession number CP000866). The full 16S rRNA and *amoA* sequences of strains HCA1^T, HCE1^T and PS0^T reported in this study were deposited under accession numbers KX950754 to KX950759.

Cell cryopreservation and resuscitation

Prior to cryopreservation, all strains were grown in standard growth medium at their optimal temperatures to late exponential phase. One-half milliliter of exponential growing culture was transferred into a 1 ml cryotube (Thermo Fisher Scientific) containing 0.5 ml of 20 % glycerol (Sigma-Aldrich). Vials were gently mixed, incubated at 20 °C for 15 min, and then preserved at -80 °C. The cryopreserved cells were resuscitated after 6 months. Frozen stocks were thawed on ice in the dark, and immediately transferred to sterile centrifuge tubes, followed by centrifugation for 45 min at 10 000 *g* at 10 °C. The supernatant with glycerol was removed to reduce toxicity and the cell pellet was gently resuspended with 1 ml standard growth medium. Subsequently, cultures were transferred into 6 ml

of fresh medium and incubated at the temperature optimum of each strain.

RESULTS AND DISCUSSION

Physiology

Growth of four strains of marine AOA was accompanied by stoichiometric conversion of ammonia to nitrite (Fig. S1, available in the online Supplementary Material) [14, 28]. The maximum cell-specific ammonia oxidation rate for strain PS0^T (2.9 fmol cell⁻¹ d⁻¹) was lower than the other three strains (5.8–12.7 fmol cell⁻¹ d⁻¹) (Table 1), but comparable to a described open ocean AOA, '*Candidatus Nitrosopelagicus brevis*' CN25 (~2 fmol cell⁻¹ d⁻¹) [55]. These values were generally within the range of the estimated per cell ammonia oxidation rate for field surveys in the Eastern Tropical North Pacific Ocean (0.1–4.1 fmol cell⁻¹ d⁻¹) and off the California coast (0.2–15 fmol cell⁻¹ d⁻¹) [18, 56]. Their physiologies differed significantly with respect to the distinct adaptations to temperature, pH, salinity, light, organic acids, and tolerance to elevated concentrations of ammonia and nitrite. The temperature optima of strains HCA1^T, HCE1^T and PS0^T were between 25 and 26 °C, whereas strain SCM1^T grew optimally at the higher temperature of 32 °C (Fig. S2, Table 1). Notably, HCE1^T was psychrotolerant and able to grow (based on cell counts and nitrite production), albeit very slowly, at temperatures as low as 4 °C (generation time of ~65 d); while slow growth of strain SCM1^T only occurred at 15 °C (generation time of ~20 d), and no growth was observed at 10 °C (Fig. S2). All four strains of marine AOA preferred circum-neutral pH, with the highest growth rates at pH 6.8–7.3 (Table 1). Unlike the other three strains, HCE1^T ceased to grow at pH 8.1, a value within the average pH range of surface oceans (Fig. S3a). Remarkably, strain PS0^T is well adapted to low-pH, sustaining approximately 80 % of its maximum growth rate at pH 5.9 [28]. All strains have an obligate salt requirement, and grow best at 25–37 ‰ salinity (Table 1). Although three coastal AOA strains (HCA1^T, HCE1^T and PS0^T) were capable of growth at oceanic salinities, they grew well at lower salinities commonly seen in coastal waters. In particular, strain HCE1^T grew over a wide salinity range (10–40 ‰) (Fig. S3b), possibly indicating adaptation to seasonal fluctuations in coastal salinity. Strain SCM1^T was tolerant of salinities between 55 and 96 ‰, but was unable to grow at 15 ‰ salinity [23, 24, 28]. Strain SCM1^T was earlier reported to have genes necessary for the synthesis of ectoine-type compatible osmolytes [30, 57] and, accordingly, the synthesis of both ectoine and hydroxyectoine was subsequently confirmed [24]. Homologous sequences of ectoine/hydroxyectoine biosynthetic genes (*ectABCD*) were also reported in the draft genomes of halotolerant *Nitrosopumilus* species from the brine-seawater interface [58], further suggesting a role of ectoines in the salt adaptation of *Nitrosopumilus* sub-lineages. Intriguingly, these genes were not identified in the genomes of *Nitrosopumilus* species enriched from low-salinity estuary and coastal environments [e.g. in

Table 1. Main characteristics of marine *Thaumarchaeota* strains SCM1^T, HCA1^T, HCE1^T and PS0^T

Characteristic	SCM1 ^T	HCA1 ^T	HCE1 ^T	PS0 ^T
Growth temperature (°C)				
Range	15–35	10–30	4–30	10–30
Optimum	32	25	25	26
Growth pH				
Range	6.8–8.1	6.8–8.1	6.4–7.8	5.9–8.1†
Optimum	7.3	7.3	7.3	6.8
Salinity (‰)				
Range	16–55	15–40	10–40	15–40
Optimum	32–37	32	25–32	25
Maximum cell-specific ammonia oxidation rates (fmol cell ⁻¹ d ⁻¹)	12.7	6.0	5.8	2.9
Ammonium tolerance (mM)‡	10	10	1	20
Use of urea	–*	–	–	+*
B-vitamin synthesis				
B ₁ (thiamine)	+	+	+	+
B ₂ (riboflavin)	+	+	+	+
B ₆ (pyridoxin)	+	+	+	+
Ado-B ₁₂ (adenosylcobalamin)	+	+	+	+
Me-B ₁₂ (methylcobalamin)	+	+	+	+
OH-B ₁₂ (hydroxocobalamin)	+	+	+	+
B ₁₂ cell quotas (nmol B ₁₂ per mol carbon)§	2800–3500	9300–11 600	4200–5300	4700–5900
DNA G+C content (mol%)	34.2	33.0	33.1	33.4

*Symbols: +, positive; –, negative.

†Strain PS0^T maintained ~80 % of the maximum ammonia oxidation activity at pH 5.9 and the effects of lower pH on its growth were not tested in this study.

‡Ammonia tolerance represents the highest tested initial ammonia concentration at which each marine AOA strain showed detectable growth (nitrite production). See Fig. S4 for the growth curves of marine AOA isolates at different initial ammonia concentrations.

§The data of B₁₂ cell quotas were previously reported by Heal et al. [35].

‘*Candidatus Nitrosopumilus salarius*’ (formerly ‘*Candidatus Nitrosopumilus salaria*’) BD31, ‘*Candidatus Nitrosopumilus piranensis*’ D3C and ‘*Candidatus Nitrosopumilus adriaticus*’ NF5] [29, 59], highlighting genotypic differentiation within the *Nitrosopumilus* genus potentially associated with their niche diversification.

All strains displayed substantially different sensitivities to high ammonium concentration (Fig. S4, Table 1). Notably, strain HCE1^T was significantly more sensitive to ammonium than other described members of *Thaumarchaeota*, being significantly inhibited at a 1 mM initial ammonium concentration (Fig. S4a); nearly no growth was observed at 2 and 3 mM ammonium. As previously reported [19], strain SCM1^T tolerates up to 10 mM ammonium (Fig. S4b) and was completely inhibited at 20 mM ammonium [19]. Strain HCA1^T exhibited somewhat greater ammonium sensitivity than SCM1^T, showing no apparent inhibition at 1 mM ammonium, partial inhibition at 2–5 mM, and significant inhibition at 10 mM (Fig. S4c). Strain PS0^T was the most ammonium-tolerant of the four AOA strains, showing comparable growth rates (as measured by nitrite production) in the presence of 0.2–10 mM initial ammonium concentrations and remaining active at 20 mM (Fig. S4d). Growth was

completely suppressed only at 50 mM. Additionally, at higher initial ammonium concentrations, the accumulation of nitrite and/or toxic intermediates also impaired growth, potentially leading to the incomplete conversion of ammonium to nitrite (Fig. S4). Strain PS0^T and one of its close relatives ‘*Candidatus Nitrosopumilus piranensis*’ D3C can also use urea as a source of ammonia for energy production and growth (Table 1) [28, 29]. The presence of a complete *ure* gene cluster consisting of urease, urease accessory proteins, and urea transporters in the complete genome of PS0^T is consistent with a capacity for urea utilization (W. Qin and D. Stahl, unpublished). By contrast, strains HCA1^T, HCE1^T and SCM1^T do not encode urease and were incapable of growth on urea (Table 1).

Although strain SCM1^T was able to grow chemolithoautotrophically by ammonia oxidation and CO₂ fixation via a modified HP/HB pathway [15], an increase in growth rate and cell yield has been observed when supplemented with small amounts of (100 μM) simple organic molecules (pyruvate, oxaloacetate, and α-ketoglutarate) [28] (Fig. S5). However, apart from these three tricarboxylic acid cycle intermediates, none of the tested 61 different organic substrates, including small organic acids, alcohols, sugars,

amines, amino acids, vitamins, energy compounds, and complex organic compounds had a positive effect on the growth of SCM1^T (Fig. S5). Similar to strains HCA1^T and PS0^T, the pure culture of HCE1^T was established and maintained in organic carbon supplemented medium, rather than in organic carbon-free medium. Comparable growth rates of the three strains were obtained in medium supplemented with pyruvate or oxaloacetate in lieu of the previously reported α -ketoglutarate (Fig. S6). The primary role of these α -keto acids in supporting the growth of HCA1^T, HCE1^T and PS0^T is likely via peroxide detoxification [26, 60]. In contrast, no growth was observed for the three strains in organic carbon-free medium or in medium supplemented with glycolate (Fig. S6).

As predicted from genome sequences, all strains produced B-vitamins thiamine (B₁), riboflavin (B₂), and pyridoxine (B₆) (Table 1). A common feature of the available thaumarchaeotal genomes and metagenomes is the presence of cobalamin (vitamin B₁₂) biosynthetic genes. In agreement with this conserved genetic capacity, the production of adenosylcobalamin (Ado-Cbl), methylcobalamin (Me-Cbl), and hydroxocobalamin (OH-Cbl) was confirmed for all four strains (Table 1). Specifically, Ado-Cbl, an active form of B₁₂, is the cofactor of methylmalonyl-CoA mutase, a key enzyme involved in thaumarchaeotal CO₂ fixation pathway [30, 61]. Total intracellular Ado-Cbl concentration increased with the exponential growth of SCM1^T, and was the dominant form of B₁₂ in actively growing cultures, revealing the tight correlation between Ado-Cbl biosynthesis and activity of *Thaumarchaeota* (Fig. 1). Notably, all strains of marine AOA have conspicuously high B₁₂:C cell quotas, ranging from 2800 to 11 600 nmol B₁₂ per mol C (Table 1). Although our calculations were based on the upper limit of B₁₂ production of marine AOA cells under optimal growth conditions without cobalt limitation, these values generally exceed those of characterized heterotrophic bacteria (0.6–6800 nmol B₁₂ per mol C, measured by bioassay) [62]. The high cellular quotas and common capacity for B₁₂ synthesis among AOA now implicate this abundant group of marine microorganisms in the provision of B₁₂ to vitamin-dependent populations in oceanic systems [35, 61].

Strains SCM1^T and PS0^T are both adapted to life under O₂ limitation, sustaining high ammonia oxidation activity at low O₂ concentrations found in suboxic regions of oxygen minimum zones (<10 μ M O₂) [25, 26]. The K_m value for O₂ uptake of strain SCM1^T was previously reported to be 3.91 μ M [14]. Notably, these strains continued to actively oxidize ammonia and divide below 1 μ M O₂ [25, 26]. Increasing amounts of nitrous oxide (N₂O) under reduced O₂ tensions have been reported for strains SCM1^T [26, 63] and PS0^T [26]. The similar impact of O₂ availability on N₂O yield was also observed with '*Candidatus Nitrosoarchaeum koreense*' (formerly '*Candidatus Nitrosoarchaeum koreensis*') MY1 and '*Candidatus Nitrosoarchaeum limnium*' (formerly '*Candidatus Nitrosoarchaeum limnia*') SFB1 [64, 65].

However, Stieglmeier et al. [66] and Kozłowski et al. [67] found no significant difference in the N₂O yield of pure cultures of SCM1^T and *Nitrososphaera viennensis* EN76^T with varying O₂ concentration and further attributed N₂O production to the abiotic reaction catalysed by reduced iron in the medium [66, 67]. Besides the production of atmospherically active trace gas N₂O, nitric oxide (NO) was also released from SCM1^T culture during active ammonia oxidation [19]. Prior demonstration of inhibition of SCM1^T [19] and *Nitrososphaera viennensis* EN76^T [67] ammonia oxidation by addition of the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (PTIO) implicated this nitrogen free radical as a participant in a novel pathway for ammonia oxidation. In contrast, the ammonia-oxidizing bacteria (AOB) are relatively insensitive to PTIO [19]. AOA and AOB also differed in the sensitivity to linear aliphatic 1-alkynes (C₆ to C₉). For instance, low concentrations of octyne (C₈; <10 μ M) completely inhibited the two AOB *Nitrosomonas europaea* and *Nitrospira multififormis*, but had little effect on SCM1^T, *Nitrososphaera viennensis* EN76, and '*Candidatus Nitrososphaera gargensis*' Ga9.2 [68, 69]. Similarly, strains SCM1^T and HCA1^T showed high resistance to allylthiourea and nitrapyrin, which have been commonly used to inhibit ammonia oxidation by AOB [19]. In contrast, other common nitrification inhibitors, such as acetylene and diethyl dithiocarbamate, strongly inhibited both strain SCM1^T and its bacterial counterparts [19].

Morphology

The four strains are morphologically indistinguishable, sharing very similar shape and size (Fig. S7a) [28]. They are slender, straight or slightly curved rods, typically 0.15–0.26 μ m in diameter and 0.50–1.59 μ m in length. Cells divide by binary fission [28, 70]. Given a long replication phase (S phase) [70], significantly elongated cells were occasionally observed in actively growing cultures (Fig. S7b). Cells occurred singly. Motility was not observed for any strains. Genes associated with chemotaxis and archaeal flagella were identified in the complete genome of strain PS0^T (W. Qin and D. Stahl, unpublished), suggestive of potential motility, but flagella were not observed using electron microscopy (EM) for cells grown under the described conditions.

The highly ordered S-layer is a major structural feature of the cell, covering the entire cell surface [71]. S-layers are common to both *Archaea* and *Bacteria*, and thought to primarily contribute to structural rigidity and general protection [71]. The regular S-layer lattices exhibit oblique (p1 or p2), square (p4), or hexagonal (p3 or p6) symmetry, and are assembled into the highly ordered two-dimensional arrays by an entropically driven process [71]. Electron CryoTomography (ECT) was used to visualize the structural details of S-layer of SCM1^T cells. Differing from the traditional EM techniques, ECT images the S-layer in its near-native state by avoiding deformations caused by staining, drying on grids, or metal shadowing [72]. In 'side' view, the SCM1^T S-layer appears to have a serrated structure (Fig. 2a). Imaging

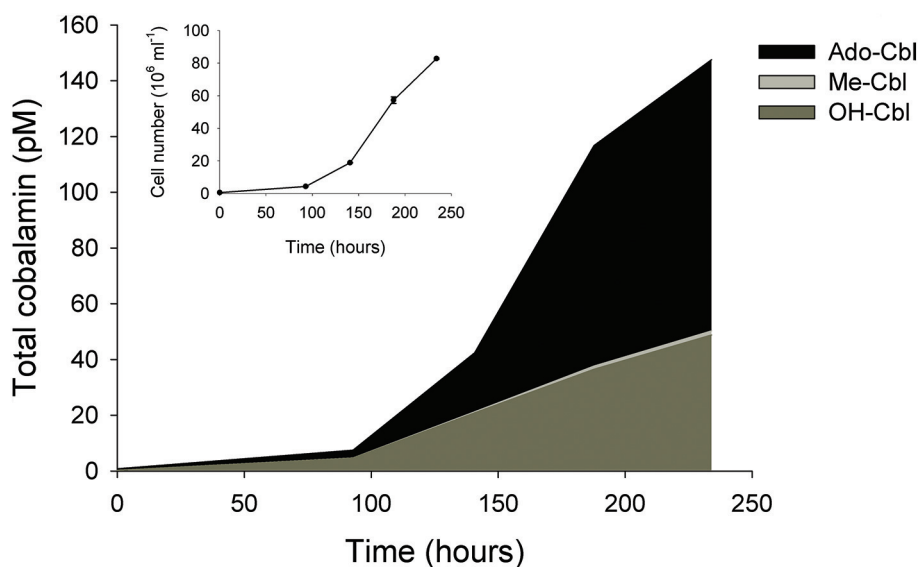


Fig. 1. Correlation of the growth of strain SCM1^T (inset panel) and total cobalamin synthesis. The individual contributions of Ado-Cbl, Me-Cbl, and OH-Cbl to total cobalamin production are shown by different shaded regions. Error bars in inset represent the standard errors of triplicate cultures. Error bars smaller than symbols are not shown.

of the surface ('Top' view) revealed a honeycomb-like lattice of regularly spaced hexagonal units (Fig. 2b). To achieve higher resolution, approximately 200 S-layer subunits were extracted from one tomogram and used to generate a 'molecular' resolution (~4 nm) structure of the S-layer lattice. The averaged subtomogram clearly showed that each S-layer structural unit consists of six protein subunits surrounding a central pore, revealing a hexagonal p6 symmetry of the S-layer lattice (Fig. 2c). The distance between two neighboring vertices of the hexagons is ~8 nm and the center-to-center distance between two hexagonal S-layer subunits is ~22 nm, within the range of typical values for S-layer (2.5–35 nm) [73]. The six-fold symmetry was further confirmed by the Fourier transform (Fig. 2d). Recently, the S-layer lattice with similar dimension constant (~21 nm) but different symmetry (p3) was reported for a group I.1b thaumarchaeote, *Nitrososphaera viennensis* EN76^T [48]. p6-S-layers are distributed over a wide range of phylogenetic branches in the domain *Archaea*, whereas p3-symmetry types have only been described for the order *Sulfolobales* besides *N. viennensis* [71]. The S-layer symmetry and lattice constant are often unique characteristics shared by members of the closely related taxa. As additional thaumarchaeotal S-layer structural information emerges, it will be of interest to assess whether a clear phylogenetic boundary exists between species with p6-S-layer and those with p3-S-layer within the phylum *Thaumarchaeota*.

Strain SCM1^T possessed a cytoplasmic membrane bounded by an S-layer that delimits a pseudoperiplasmic space (Fig. 2a). The monolayer cell membrane of strain SCM1^T is comprised of GDGT core lipids bound to glyco- or phospho- polar head groups [38]. The core lipids consisted

mainly of GDGTs with 0–4 cyclopentane rings (GDGT-0–4) and crenarchaeol, the characteristic GDGT of *Thaumarchaeota*, containing a cyclohexane ring and 4 cyclopentane rings [25, 38]. Only minor amounts of crenarchaeol regioisomer (cren') occurred in four strains of marine AOA as well as other investigated group I.1a *Thaumarchaeota* [23, 25, 36], whereas much higher proportions have been observed in group I.1b *Thaumarchaeota* [37, 74]. Hydroxylated GDGTs were observed exclusively in group I.1a and the associated group, but not in group I.1b *Thaumarchaeota* [34, 37]. In addition, although GDGTs with a trihexose headgroup occurred in soil *Thaumarchaeota* '*Candidatus Nitrosotalea devanaterre*' Nd1 and *Nitrososphaera viennensis* EN76^T [34, 37], they have not been detected in any marine *Thaumarchaeota* analysed so far. These marked differences together suggest that the core and intact polar GDGT compositions in *Thaumarchaeota* likely reflect both phylogenetic distributions and ecological niche differentiation. The membrane lipid composition of *Nitrosopumilus* species was recently shown to be influenced by O₂ concentration, showing an apparent increase of GDGT-2 at the expense of GDGT-0 and GDGT-1 at lower O₂ tensions [25]. The core and intact polar GDGTs of strain SCM1^T also changed with growth phase [38]. Subsequent work on lipid compositional changes in response to ammonia oxidation rate supported the suggestion by Elling *et al.* [38] that energy and reducing power limitation leads to shifts in core GDGT composition, hypothesizing that GDGT cyclization is related to energy flow through the cell [75]. In contrast, changes in salinity and pH had no significant impact on membrane lipid composition [23].

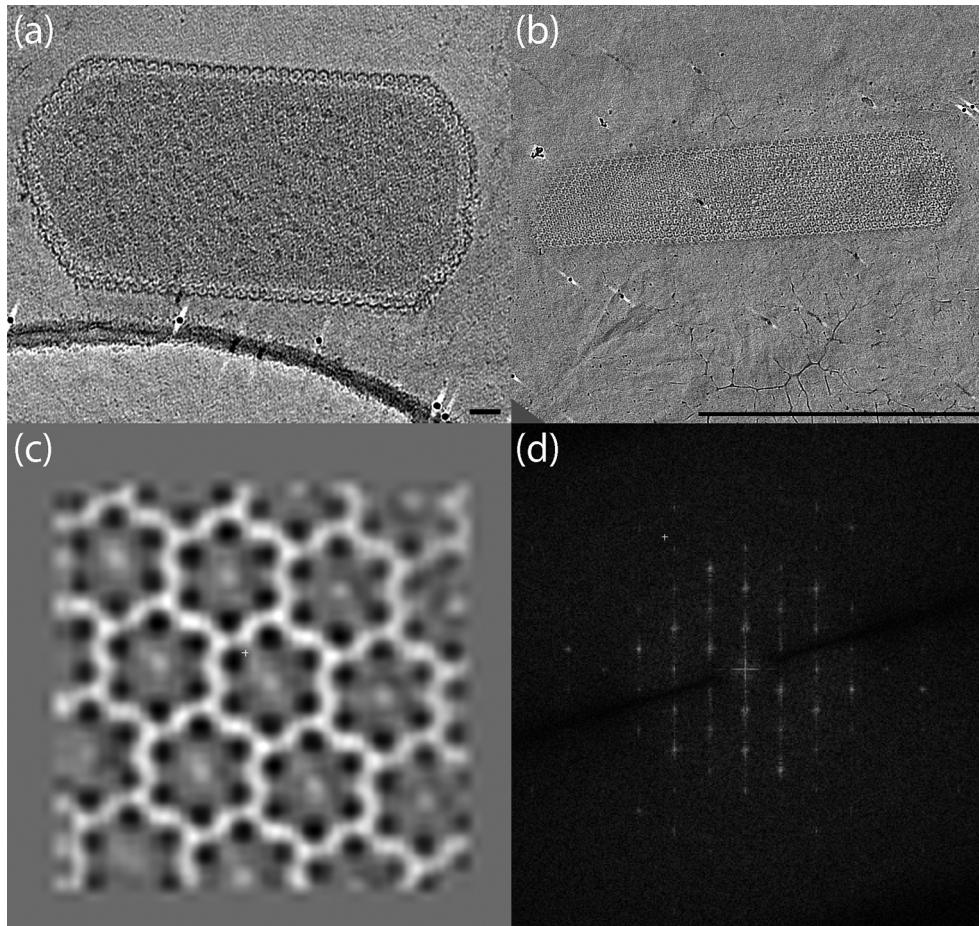


Fig. 2. S-layer structure of strain SCM1^T. (a) Side view of S-layer showing serrated pattern. (Scale bar: 50 nm) (b) Surface view of S-layer showing honeycomb-like pattern. (Scale bar: 1 μ m) (c) Subtomogram averages of S-layer showing hexagonal subunits in p6-symmetry. (d) Fourier transform of the subtomogram.

Phylogeny

Although the four strains of marine AOA displayed distinct physiological characteristics and metabolic capacities, they shared close phylogenetic relationships, united by ~98 % 16S rRNA and ~94 % *amoA* gene sequence identity. Phylogenetic analysis of 16S rRNA and *amoA* genes placed these strains within a highly supported monophyletic clade tightly associated with other marine group I.1a *Thaumarchaeota* and distinct from the low-salinity and fresh water AOA lineages (Figs 3 and S8). They shared less than 94 and 85 % identity with the 16S rRNA and *amoA* genes, respectively, with other deeply-branching groups of group I.1a *Thaumarchaeota*, including ‘*Candidatus Nitrosopelagicus brevis*’ CN25, ‘*Candidatus Cenarchaeum symbiosum*’ A, ‘*Candidatus Nitrosotenuis uzonensis*’ N4 and ‘*Candidatus Nitrosotenuis chungbukensis*’ MY2. Similarly, sequences of their 16S rRNA and *amoA* genes were more than 11 and 21 % divergent, respectively, from those of other major sublineages of *Thaumarchaeota* including group I.1a-associated, group I.1b, and ThAOA. Consistent with most

mesophilic marine and lacustrine *Thaumarchaeota*, all four marine AOA strains have a low mol% G+C content of 33.0–34.2 (Table 1), which are ~3 % lower than that determined for the soil acidophilic strain ‘*Candidatus Nitrosotalea devanattera*’ Nd1 (group I.1a-associated), ~8 % lower than that of a moderately thermophilic strain ‘*Candidatus Nitrosotenuis uzonensis*’ N4 (group I.1a), and >14 % lower than group I.1b strains ‘*Candidatus Nitrososphaera gargensis*’ Ga9.2 (48.3 %), ‘*Candidatus Nitrososphaera evergladensis*’ SR1 (50.1 %) and *Nitrososphaera viennensis* EN76^T (52.7 %). Despite high similarities of G+C content and the sequences of two phylogenetically informative genes, their whole genomes shared less than 84 % average nucleotide identity (ANI) (W. Qin and D. Stahl, unpublished). These values are far below the threshold range (95–96 %) now generally accepted for species definition [76]. Therefore, in addition to *Nitrosopumilus maritimus* sp. nov. strain SCM1^T, we propose that strains HCA1^T, HCE1^T and PS0^T be assigned to three novel species as *Nitrosopumilus cobalaminigenes* sp. nov., *Nitrosopumilus oxyclinae* sp. nov., and *Nitrosopumilus ureiphilus* sp. nov., respectively.



Fig. 3. Maximum-likelihood tree based on the 16S rRNA sequences showing the phylogenetic relationships of the order *Nitrosopumilales*, the family *Nitrosopumilaceae*, and the genus *Nitrosopumilus*. Confidence values are on the basis of 500 bootstrap replications. The scale bar represents 1% estimated sequence divergence. Please note that the original names, '*Candidatus Nitrosopumilus koreensis*', '*Candidatus Nitrosopumilus salaria*', '*Candidatus Nitrosoarchaeum limnia*', '*Candidatus Nitrosoarchaeum koreensis*', and '*Candidatus Nitrosocaldus yellowstonii*' have been corrected as '*Candidatus Nitrosopumilus koreense*', '*Candidatus Nitrosopumilus salarius*', '*Candidatus Nitrosoarchaeum limnium*', '*Candidatus Nitrosoarchaeum koreense*', and '*Candidatus Nitrosocaldus yellowstonensis*', respectively, as recommended in the International Code of Nomenclature of Prokaryotes [45–47, 82].

DESCRIPTION OF *NITROSOPUMILUS* GEN. NOV.

Nitrosopumilus (Ni.tro.so.pu'mi.lus. L. adj. *nitrosus*, full of natron; here intended to mean nitrous; L. masc. n. *pumilus* dwarf; N.L. masc. n. *Nitrosopumilus* a dwarf producing nitrite).

Cells are straight or slightly curved rods 0.49–2.00 μm in length and 0.15–0.27 μm in width. Occurring singly. Non-motile or motile by polar to subpolar flagella [29]. Cell envelope consists of a hexagonally arrayed single S-layer and a monolayer cytoplasmic membrane containing GDGT lipids with 0 to 5 cycloalkyl moieties. The major GDGT membrane lipid, crenarchaeol, contains four cyclopentyl moieties and one cyclohexyl moiety. The respiratory quinones are saturated and monounsaturated menaquinones with 6 isoprenoid units [39]. Aerobic. Chemolithoautotrophic growth by ammonia oxidation to nitrite using CO_2 as carbon source, although some organic acids may be needed for growth. Many, but not all, species also use urea as a source of ammonia for energy production and growth. Their apparent half-saturation constants (K_m) for O_2 uptake and ammonia oxidation are 1.17–3.91 μM and 0.13–0.61 μM total ammonia ($\text{NH}_3 + \text{NH}_4^+$), respectively [14, 77]. Cells tolerate up to 20 mM ammonium. Mesophilic, optimal growth temperature between 25 and 32 $^\circ\text{C}$. Neutrophilic, pH optimum between 6.8 and 7.3. Slight to moderate halophilic, salinity optimum between 25 and 37 ‰. Cells are sensitive to light. Members are capable of vitamin B₁, B₂, B₆ and B₁₂

synthesis. Occur free-living in a wide range of marine systems, including surface oceans, hadal oceans, saltwater aquaria, brackish waters, marine and estuarine sediments, salt marshes, and brine-seawater interfaces. Phylogenetic analyses of 16S rRNA and *amoA* sequences indicate that species of the genus *Nitrosopumilus* form a highly supported monophyletic lineage within the group I.1a *Thaumarchaeota*. Their closest relatives affiliate with the provisional genus '*Nitrosoarchaeum*' [represented by '*Candidatus Nitrosoarchaeum koreense*' (formerly '*Candidatus Nitrosoarchaeum koreensis*') MY1, '*Candidatus Nitrosoarchaeum limnium*' (formerly '*Candidatus Nitrosoarchaeum limnia*') BG20 and SFB1] commonly found in low-salinity and freshwater environments [64, 65]. The DNA G+C content is 33.0–34.2 mol%. The type species is *Nitrosopumilus maritimus*.

DESCRIPTION OF *NITROSOPUMILUS* *MARITIMUS* SP. NOV.

Nitrosopumilus maritimus (ma.ri'ti.mus. L. masc. adj. *maritimus* belonging to the sea; describing its habitat).

Displays the following properties in addition to those given in the genus description. Slender rods with a length of 0.50–0.90 μm and a diameter of 0.17–0.22 μm . Motility is not observed. The cell envelope consists of an S-layer with p6 symmetry covering a monolayer cytoplasmic membrane. The apparent K_m values of O_2 uptake and ammonia oxidation are $3.91 \pm 0.57 \mu\text{M}$ and $0.133 \pm 0.038 \mu\text{M}$, respectively.

Cells grow better at low O₂ concentrations of 5–10 % head-space O₂ than 21 % O₂. Cells tolerate ammonia and nitrite concentrations of up to 10 mM and 2 mM, respectively. Urease negative. Growth occurs between 15 and 35 °C, with an optimum of 32 °C. The pH range for growth is 6.8–8.1, with an optimum of pH 7.3. The salinity range for growth is 16–96 ‰, with an optimum of 32–37 ‰ salinity. The minimum generation time is around 19 h. Cells are photosensitive and completely inhibited by continuous illumination at a light intensity of 120 µE m⁻² s⁻¹ [28]. Both ectoine and hydroxyectoine are synthesized in cells in response to osmotic shock [24]. Cells are capable of B₁₂ synthesis with the B₁₂:C cell quotas of 2800–3500 nmol B₁₂ per mol cellular carbon.

The type strain is SCM1^T(=ATCC TSD-97^T=NCIMB 15022^T), isolated from a tropical marine fish tank at the Seattle Aquarium in Seattle, Washington, USA. The G+C content of the genomic DNA of the type strain is 34.2 mol%.

DESCRIPTION OF *NITROSOPUMILUS COBALAMINIGENES* SP. NOV.

Nitrosopumilus cobalaminigenes (co.bal.a.mi.ni'ge.nes. N.L. neut. n. *cobalaminum* cobalamin; Gr. v. *gennao* to make, to produce; N.L. masc. adj. *cobalaminigenes* cobalamin producing, referring to the high cobalamin cell quotas of the type strain).

Displays the following properties in addition to those described above for the genus. Cells are straight small rods 0.65–1.27 µm in length and 0.15–0.26 µm in width. Non-motile. Urease negative. Optimum growth occurs with 0.2–1 mM ammonium and ammonium tolerance is up to 10 mM at pH 7.5. Optimal growth temperature: 25 °C; range, 10–30 °C. Optimal pH: 7.3; range, 6.8–8.1. Optimal salinity: 32 ‰; range, 15–40 ‰ salinity. The minimum doubling time is around 30 h. Cells are sensitive to light and completely inhibited by cycles of 14 h dark/10 h light at a light intensity of 180 µE m⁻² s⁻¹ [28]. B₁₂ is synthesized at a high carbon specific cell quota level, ranging from 9300 to 11600 nmol B₁₂ per mol cellular carbon.

The type strain is HCA1^T (=ATCC TSD-96^T), obtained from a depth of 50 m seawater at the Puget Sound Regional Synthesis Model Station P10 (47.91 N, 122.62 W) in Hood Canal, Washington, USA. The G+C content of the genomic DNA of the type strain is 33.0 mol%.

DESCRIPTION OF *NITROSOPUMILUS OXYCLINAE* SP. NOV.

Nitrosopumilus oxycliniae (o.xy.cli'nae. Gr. adj. *oxys* sharp/acid; Gr. v. *clinein* decline; N.L. gen. n. *oxycliniae* referring to the oxycline from where the type strain was isolated).

Slender rods, 0.17–0.26 µm × 0.69–0.93 µm. Non-motile. Urease negative. Cells are sensitive to relatively high concentrations of ammonium with an ammonium tolerance of up to 1 mM at pH 7.5. Psychrotolerant, growing between 4

and 30 °C, with optimum growth at 25 °C. Growth occurs at pH 6.4–7.8 (optimum pH 7.3) and at 10–40 ‰ salinity (optimum 25 ‰). The minimum doubling time is around 33 h. Cells are capable of B₁₂ synthesis with the B₁₂:C cell quotas of 4200–5300 nmol B₁₂ per mol cellular carbon. Other general characteristics are the same as those described for the genus.

The type strain is HCE1^T (=ATCC TSD-98^T), isolated from a depth of 17 m seawater (nitrite maximum) within the oxycline of the Puget Sound Regional Synthesis Model Station P12 (47.42 N, 123.11 W) in Hood Canal, Washington, USA. The G+C content of the genomic DNA of the type strain is 33.1 mol%.

DESCRIPTION OF *NITROSOPUMILUS UREIPHILUS* SP. NOV.

Nitrosopumilus ureiphilus (u.re.i'phi.lus. N.L. n. *urea* urea; Gr. adj. *philos* loving; N.L. masc. adj. *ureiphilus* loving urea).

Straight, or slightly curved rods, 0.22–0.26 µm × 0.76–1.59 µm. Non-motile, although the presence of genes for an archaeal flagellum and chemotaxis indicate cells may be motile under some conditions. Urea can serve as a source of ammonia for energy generation and growth. The maximum ammonium tolerance is 20 mM at pH 7.5. Cells grow best at ambient O₂ concentration (21 % O₂), but still sustain high ammonia oxidation activity at O₂ concentrations lower than 10 µM and continue to actively oxidize ammonia and divide at sub-micromolar O₂. Growth occurs between 10–30 °C (optimum 26 °C) and at 15–40 ‰ salinity (optimum 25–32 ‰). The optimal pH is 6.8, but cells grow well at a pH as low as 5.9. The minimum doubling time is around 54 h. Cells are light sensitive and completely inhibited by cycles of 14 h dark/10 h light at a light intensity of 80 µE m⁻² s⁻¹ [28]. They produce B₁₂ with the carbon specific quotas of B₁₂ ranging from 4700 to 5900 nmol B₁₂ per mol cellular carbon. Otherwise, the description is the same as that for the genus.

The type strain is PS0^T (=ATCC TSD-99^T), originated from a near-shore surface sediment (47.59 N, 122.40 W) in Puget Sound near Seattle, Washington, USA. The G+C content of the genomic DNA of the type strain is 33.4 mol%.

DESCRIPTION OF *NITROSOPUMILACEAE* FAM. NOV.

Nitrosopumilaceae (Ni.tro.so.pu.mi.la.ce'ae. N.L. masc. n. *Nitrosopumilus* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Nitrosopumilaceae* the family of the genus *Nitrosopumilus*).

Mesophilic, neutrophilic, motile or nonmotile, free-living or symbiotic, straight or slightly curved rods. Obligatorily aerobic ammonia-oxidizing archaea; some members also use urea as substrate. Autotrophic, fixing CO₂ by a HP/HB cycle, although some organic material may be needed to support growth. Found in a variety of habitats, including

marine, freshwater, wastewater, and soil. Members of this family comprise a distinct branch within the group I.1a *Thaumarchaeota* based on both 16S rRNA and *amoA* sequences analyses. In contrast to the provisional family 'Nitrosotenuaceae' (represented by 'Candidatus Nitrosotenuis uzonensis' N4, 'Candidatus Nitrosotenuis chungbukensis' MY2, and 'Candidatus Nitrosotenuis cloacae' SAT1) [33, 78, 79], their growth does not occur under moderately thermophilic condition. So far, the family comprises the genus *Nitrosopumilus*, 'Candidatus Nitrosoarchaeum', 'Candidatus Nitrosopelagicus' (represented by 'Candidatus Nitrosopelagicus brevis' CN25) [31] and 'Candidatus Cenarchaeum' (represented by 'Candidatus Cenarchaeum symbiosum' A) [80]. The type genus is *Nitrosopumilus*. See also description of the genus for features.

DESCRIPTION OF NITROSOPUMILALES ORD. NOV.

Nitrosopumilales (Ni.tro.so.pu.mi.la'les. N.L. masc. n. *Nitrosopumilus* type genus of the order; -ales ending to denote an order; N.L. fem. pl. n. *Nitrosopumilales* the order of the genus *Nitrosopumilus*).

Slender rods or irregular cocci. Obligate aerobes. Chemolithoautotrophs, using ammonia as energy source and CO₂ as carbon source, although some organic material may be needed for growth. Membrane lipids contain acyclic and cyclized GDGTs with up to 5 cycloalkyl rings. Crenarchaeol is a major core GDGT, while its regioisomer (cren') is detected at low abundance. Hydroxylated GDGTs are exclusively observed for *Thaumarchaeota* affiliated to this order. Cells contain saturated and monounsaturated menaquinones with 6 isoprenoid units in the side chain. Mesophilic to moderately thermophilic. Neutrophilic. Free-living or symbiotic. All investigated genomes of the members of this order possess cobalamin biosynthesis genes, suggesting that the production of B₁₂ might be common within the *Nitrosopumilales* order. The order *Nitrosopumilales* refers to the group I.1a *Thaumarchaeota*, which is phylogenetically distinct from the orders 'Candidatus Nitrosotaleales' (refers to group I.1a-associated; represented by 'Candidatus Nitrosotalea devanaterra' Nd1) [81], *Nitrososphaerales* (refers to the group I.1b) [48] and 'Candidatus Nitrosocaldales' (refers to the ThAOA group; represented by 'Candidatus Nitrosocaldus yellowstonensis' (formerly 'Candidatus Nitrosocaldus yellowstonii') HL72) [21] within the class *Nitrososphaeria*. Occur worldwide in marine, terrestrial, and geothermal habitats. This order contains the families *Nitrosopumilaceae* and 'Candidatus Nitrosotenuaceae'. The type genus is *Nitrosopumilus*.

Funding information

This work was funded by the United States National Science Foundation grants MCB-0604448 (to D. A. S.) and Dimensions of Biodiversity Program OCE-1046017 (to D. A. S., A. E. I., E. V. A., A. H. D., J. W. M.).

Acknowledgements

We thank B. Schneider and the FHCRC EM staff for performing transmission electron microscopy, and the Captain and crew of the R/V Clifford A. Barnes for their assistance with sample collection. We thank

Adam Gee, Emily Chang, Yue Zheng, Jessie Zhou, Dr Robert Morris, and Vega Shah for technical assistance and Andrea Teichgräber and Kelley Meinhardt for helpful discussions. Finally, we thank the editor, Dr Aharon Oren, for particularly helpful comments and suggestions.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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