**Nitrososphaera viennensis** gen. nov., sp. nov., an aerobic and mesophilic, ammonia-oxidizing archaeon from soil and a member of the archaeal phylum *Thaumarchaeota*

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A mesophilic, neutrophilic and aerobic, ammonia-oxidizing archaeon, strain EN76T, was isolated from garden soil in Vienna (Austria). Cells were irregular cocci with a diameter of 0.6–0.9 μm and possessed archaella and archaeal pili as cell appendages. Electron microscopy also indicated clearly discernible areas of high and low electron density, as well as tubule-like structures. Strain EN76T had an S-layer with p3 symmetry, so far only reported for members of the *Sulfolobales*. Crenarchaeol was the major core lipid. The organism gained energy by oxidizing ammonia to nitrite aerobically, thereby fixing CO₂, but growth depended on the addition of small amounts of organic acids. The optimal growth temperature was 42 °C and the optimal pH was 7.5, with ammonium and pyruvate concentrations of 2.6 and 1 mM, respectively. The genome of strain EN76T had a DNA G+C content of 52.7 mol%. Phylogenetic analyses of 16S rRNA genes showed that strain EN76T is affiliated with the recently proposed phylum *Thaumarchaeota*, sharing 85% 16S rRNA gene sequence identity with the closest cultivated relative ‘*Candidatus Nitrosopumilus maritimus*’ SCM1, a marine ammonia-oxidizing archaeon, and a maximum of 81% 16S rRNA gene sequence identity with members of the phyla *Crenarchaeota* and *Euryarchaeota* and any of the other recently proposed phyla (e.g. ‘*Korarchaeota*’ and ‘*Aigarchaeota*’). We propose the name *Nitrososphaera viennensis* gen. nov., sp. nov. to accommodate strain EN76T. The type strain of *Nitrososphaera viennensis* is strain EN76T (DSM 26422T = JMC 19564T).

Additionally, we propose the family *Nitrososphaeraceae* fam. nov., the order *Nitrososphaerales* ord. nov. and the class *Nitrososphaeria* classis nov.

**Introduction**

Microbes are immensely diverse and abundant, and inhabit virtually all environments on Earth. However, most of this microbial diversity remains undescribed, given that many novel organisms are fastidious and their isolation and cultivation is time-consuming or even impossible (Rappé & Giovannoni, 2003). Cultivation-independent techniques have increased our knowledge of microbial diversity and metabolism tremendously (Lane *et al.*, 1985; Marcy *et al.*, 2007; Rinke *et al.*, 2013; Schmidt *et al.*, 1991; Stein *et al.*, 1996), and have led to the proposal of several new bacterial and archaeal phyla (e.g. Elkins *et al.*, 2008; Gordon & Giovannoni, 1996; Huber *et al.*, 2002; Hugenholtz *et al.*, 1998; Nunoura *et al.*, 2011; Rinke *et al.*, 2013). In addition, metabolic predictions based on meta-omic studies can also support the design of media and consequently facilitate the cultivation of uncharacterized microorganisms (Tyson & Banfield, 2005). When used in combination, microbial cultivation and metagenomics represent a powerful toolbox to describe newly discovered microbial physiologies, and to simultaneously assess their ecological distribution and impact. The discovery of ammonia-oxidizing archaea...
Nitrososphaera viennensis gen. nov., sp. nov.

(Alveoarchaeota) represents a successful example of such an integrated approach. These archaea, originally called ‘mesophilic Crenarchaeota’, were first identified in marine samples (DeLong, 1992; DeLong et al., 1994; Fuhrman et al., 1992) and were later detected in many more environments (Jurgens et al., 1997; Karner et al., 2001; MacGregor et al., 1997; Ochsenreiter et al., 2003; Preston et al., 1996; Schleper et al., 1997; Takai et al., 2001). The first evidence for their metabolism and potential ecological role was given by the discovery of genes encoding a putative ammonia mono-oxygenase, the key enzyme for ammonia oxidation, through metagenomics (Hallam et al., 2006b; Treusch et al., 2005; Venter et al., 2004) and through the cultivation and isolation of the first AOA, ‘Candidatus Nitrosopumilus maritimus’ SCM1 (Könneke et al., 2005). The widespread occurrence of potential archaean ammonia oxidizers in the environment has been confirmed by a large number of qualitative and quantitative molecular surveys (Adair & Schwartz, 2008; Alves et al., 2013; de la Torre et al., 2008; Francis et al., 2005; He et al., 2007; Hershberger et al., 1996; Leininger et al., 2006; Pester et al., 2012; Reigstad et al., 2008; Shen et al., 2008; Wuchter et al., 2006; Zhang et al., 2008). Based on phylogenetic analyses of concatenated ribosomal protein sequences and full-genome comparisons with the first genome sequence of the putative AOA ‘Candidatus Cenarchaeum symbiosum’ (Hallam et al., 2006a), Brochier-Armanet et al. (2008) suggested that the AOA comprise a new archeal phylum, the Thaumarchaeota, which was subsequently supported by Spang et al. (2010) upon the inclusion of two more genome sequences of members of the new phylum.

Due to the difficulty in cultivating and purifying members of the Thaumarchaeota in laboratory cultures, pure isolates are still rare (Könneke et al., 2005; Tourn et al., 2011) and insights into the physiology of AOA are confined to studies of two pure cultures (Könneke et al., 2005; Martens-Habbena et al., 2009; Schouten et al., 2008) or are based on enrichment cultures (de la Torre et al., 2008; Hatzenpichler et al., 2008; Jung et al., 2011; Lehtovirta-Morley et al., 2011; Santoro & Casciotti, 2011). ‘Candidatus Nitrosopumilus maritimus’ SCM1 is a representative of the marine I.1a group, which is one of the distinct lineages formed within the phylum Thaumarchaeota. With the isolation of strain EN76, the first pure culture from soil and group I.1b was obtained, which represents the second major lineage of the Thaumarchaeota. The isolation of strain EN76 confirmed ammonia oxidation by thaumarchaeotes in soil and expanded the metabolic spectrum of AOA to include the utilization of urea and organic substrates (Tourn et al., 2011). These findings demonstrate the importance of pure cultures to the investigation of growth requirements, and will also help in future cultivation and purification experiments of other members of the Thaumarchaeota.

The open ocean, marine sediments and soil contain large numbers of microbes, with approximately $1.2 \times 10^{29}$, $2.9 \times 10^{29}$ and $2.6 \times 10^{29}$ cells, respectively (Kallmeyer et al., 2012; Whitman et al., 1998). Molecular surveys based on amoA or 16S rRNA gene sequences have shown that members of the Thaumarchaeota make up a large fraction of the microbial biomass in all three habitats (Bates et al., 2011; Karner et al., 2001; Leininger et al., 2006). With up to 20% of the picoplankton in the marine environment (Karner et al., 2001), up to 80% of the microbiota in certain horizons of marine sediments (Durbin & Teske, 2010; Jorgensen et al., 2012) and up to 1% of the total microbiota in soil (Bates et al., 2011; Leininger et al., 2006), the total abundance of thaumarchaeetes seems to be comparable to that of other highly abundant bacterial phyla, even to that of the ubiquitous Proteobacteria. Based on their large numbers in many environments and their ability to perform the first and rate-limiting step in nitrification, the oxidation of ammonia to nitrite, members of the Thaumarchaeota are now considered to play a major role in the global nitrogen cycle (Alves et al., 2013; Di et al., 2009; Erguder et al., 2009; Gubry-Rangin et al., 2010; Hatzenpichler, 2012; Jia & Conrad, 2009; Nicol & Schleper, 2006; Offre et al., 2009; Prosser & Nicol, 2012; Schauss et al., 2009; Schleper & Nicol, 2010; Stahl & de la Torre, 2012; Stieglmeier et al., 2014a; Wuchter et al., 2006; Xia et al., 2011; Zhalnina et al., 2012). Several factors have been suggested as drivers of environmental adaptation and niche selection of thaumarchaeotes, such as pH and ammonia concentration (reviewed by Prosser & Nicol, 2012). For example, ‘Candidatus Nitrosopumilus maritimus’ SCM1 and other marine members of the Thaumarchaeota have been shown to have a higher affinity to ammonia than cultivated ammonia-oxidizing bacteria (AOB), and all strains in pure culture or enrichment are adapted to low concentrations of ammonia (Horak et al., 2013; Martens-Habbena et al., 2009; Prosser & Nicol, 2012). However, thaumarchaeotes are widespread in diverse habitats, exposed to wide ranges of pH and ammonia concentration, and recent studies suggest that they almost certainly harbour a broader range of physiological and/or metabolic properties than is currently known (Alves et al., 2013; Durbin & Teske, 2010; Jorgensen et al., 2012; Muller et al., 2010; Mussmann et al., 2011). In order to understand their ecological impact, it is important to identify and characterize in detail the metabolic functions and physiological versatility of more representatives of these archaea.

Although AOA have been studied intensively in the last decade (reviewed by e.g. Nicol & Schleper, 2006; Stahl & de la Torre, 2012; Stieglmeier et al., 2014a), there are presently no validly published names of species, genera or higher ranks within the phylum Thaumarchaeota. Here, we extend the original characterization of strain EN76 (Tourna et al., 2011), with a special focus on its ultrastructure and growth improvements, and formally propose the species Nitrososphaera viennensis sp. nov. and propose to assign this species as the type species of the genus Nitrososphaera gen. nov. The genus Nitrososphaera is the type of the family Nitrososphaeraceae fam. nov. and the order Nitrososphaerales ord. nov., which is in turn the type of the class Nitrososphaerida class nov.
Methods

Sample source and culture conditions. Sample source and isolation strategy for strain EN76T were described previously by Touna et al. (2011). In brief, strain EN76T was isolated from Viennese garden soil (48°13' 48.72" N 16°21' 28.93" E) by transferring 5 g soil into 50 ml freshwater medium (FWM) containing (1 g NaCl, 0.4 g MgCl2 6H2O, 0.1 g CaCl2, 2H2O, 0.2 g KH2PO4, 0.5 g KCl, 1 ml trace element mixture, 1 ml vitamin solution and 7.5 μM ferric sodium EDTA, 0.5 mM NH4Cl as energy source and 2 mM NaHCO3 as carbon source. Additionally 0.1 mM NaN3 was supplied to the cultures. The medium was adjusted to pH 7.5 and cultures were incubated at 37 °C in the dark without shaking. Supplementation with antibiotics (carbenicillin, streptomycin) and filtration of the cultures (0.45 μm pore size) were applied to reduce bacterial and fungal contaminants. Growth was followed by measuring ammonium consumption and nitrite production photometrically. Additionally, light microscopy and quantitative PCR were used as described previously (Touna et al., 2011).

The purified strain EN76T was routinely cultured in 20 ml FWM in sterile 30 ml polystyrene screw-capped containers (VWR; catalogue no. 216-2637) supplemented with 1 mM NH4Cl, 2 mM NaHCO3, 0.1 mM sodium pyruvate and 100 μg antibiotics ml−1 (kanamycin, streptomycin or oloxacin in MilliQ water). The medium was buffered with HEPES/NaOH to pH 7.5. Larger volumes were cultured in glass bottles and shaken (150 r.p.m.) in darkness.

Physiological characterization and multivariate optimization of growth conditions. To determine optimal growth parameters as well as the effect of different nitrogen and carbon substrates on the growth of EN76T, the strain was cultivated in closed batch in 20 ml FWM containing 100 μg antibiotics ml−1 as described above. Substrates were dissolved in MilliQ water and sterile filtered (0.2 μm) before usage. Table 1 gives an overview of tested substrates.

In order to investigate optimal growth conditions for EN76T, a design of experiments (DoE) strategy was applied, using the factors temperature, pyruvate concentration and ammonium concentration. Based on our preliminary knowledge of the strain’s growth requirements (Touna et al., 2011), the range for each factor (design space) was set as follows: 37–47 °C, 0.1–1.5 mM sodium pyruvate and 1–4 mM NH4Cl. As nitrite production was shown to follow biomass production (Touna et al., 2011), it was used to calculate the growth rate (μ) and maximum growth rate (μmax), which were eventually used to develop the model (Design-Expert 8 software; Stat-Ease Inc.). Experiments were conducted in triplicate, except for the centre points of the initial two-level factorial screening design, which were set up in fivefold replicates. The two-level factorial design was applied in order to screen the design space rapidly. Due to a low model significance of data obtained from the initial two-level factorial screening design space, an augmented matrix was used in order to account for putative interactions of individual factors. Thus, the two-level factorial design space was extended by using a face-centred augmented matrix. Eventually, data points of all experiments (n=51) were used to establish a response surface model (RSM). Data were analysed with the software Design-Expert 8. ANOVA, based on a stepwise regression elimination procedure, was used to develop the model. The desirability approach, as described elsewhere (Derringer & Suich, 1980), was used to maximize μ or μmax (variable) based on variation of quantitative factors, here (ammonium) and (pyruvate) and temperature (within the design space). A score is given to each quantitative factor setting that can be used to maximize the variable. In this approach, desirability between 0 and 1 (corresponding to 0–100%) can be assigned to a variable for optimization; factors identified as being outside a certain desirability function will not be considered for model generation. To verify the calculated optimal growth conditions identified by the established RSM model design space, one additional growth experiment (fivefold-replicated closed-batch cultures) was performed (Fig. 1a).

Microscopy. For negative staining, cells were fixed with 2.5% glutaraldehyde in 1 × PBS, transferred to carbon-coated copper grids (200 mesh) and stained with 0.5% uranyl acetate for 2 min as described previously (Touna et al., 2011). Images were recorded with a megaview III camera (SIS) attached to a Philips EM 208 transmission electron microscope (FEI) operated at 70 keV.

For scanning electron microscopy (SEM), poly-L-lysine-coated glass slides were added to the culture from early to late exponential phase. Attached cells were fixed for 2 h at room temperature with 2% glutaraldehyde and 2% formaldehyde in 0.06 × PHEM buffer (buffer based onPIPES, HEPES, EGTA and MgCl2; J. Montanaro and N. Leisch, unpublished). The cells were post-fixed with 1% osmium tetroxide for 2 h at room temperature followed by dehydration in a graded ethanol series. After immersion in pure acetone, the slides were critical-point-dried with a CPD 300 unit (Leica). The slides were then mounted on stubs, gold-coated with an AGAR B7340 spatter-coater and imaged using an XL200 instrument (Philips) running the microscope control program (version 7.00; FEI).

For the preparation of ultrathin sections, cells were grown in 1 l FWM supplemented with 3 mM NH4Cl and 0.15 mM sodium pyruvate until the late exponential growth phase. Cells were harvested by centrifugation and fixed as mentioned above for SEM preparation. After fixation, cells were washed twice in 100 mM PHEM buffer and covered with 1% agar. Cells were post-fixed for 1 h in osmium tetroxide (1%), washed three times (1 × PHEM; J. Montanaro and N. Leisch, unpublished), dehydrated in a graded ethanol series and embedded in resin with acetonitrile as solvent. Polymerization was achieved by incubating the resin blocks for 1 h at 40 °C and for 48–72 h at 60 °C (Leisch et al., 2011). An Ultracut S (Leica) was used to produce ultrathin sections (70 nm), which were then transferred to copper grids (300 mesh). They were post-stained with uranyl acetate and lead citrate before visualization on a Zeiss 902 instrument equipped with an Olympus SharpEye camera at an accelerating voltage of 80 kV and a Libra120 instrument (Carl Zeiss) equipped with a slow-scan CCD camera (Tröndle) at an accelerating voltage of 120 kV. Images were post-processed with Adobe Photoshop CS5.

To determine the general morphology and properties of the potential S-layer protein of EN76T, negative staining of purified S-layer sheets as well as freeze-fracturing/freeze-etching was performed. If not otherwise mentioned, freeze-etching was carried out as described previously (Klingl et al., 2011; Rachel et al., 2010). The purification of S-layer proteins was done by breaking the cells by sonication, differential centrifugation and extraction of lipids, using a MES buffer system (Klingl, 2011; Veith et al., 2009). Subsequent negative staining of S-layer sheets for transmission electron microscopy (TEM) was performed as described previously (Rachel et al., 2010). For the investigation of freeze-etching replicas and purified S-layer proteins, a JEOL JEM 2100 TEM, equipped with a fast scan 2k × 2k CCD camera F214 (TVIPS), was used at an accelerating voltage of 120 kV. Image analyses, correlation averaging and determination of S-layer symmetry and lattice constants of negatively stained S-layer proteins as well as freeze-etching replicas was performed with the ANIMETRA CRYSTALS software package (release 1.1; Animetra).

Lipid analyses. Intact polar lipids and glycerol dibiphytanyl glycerol tetraether (GDGT) were analysed previously and described by Sinninghe Damsté et al. (2012).
Table 1. Effect of substrates on growth of strain EN76<sup>T</sup>

The effect of various substrates on growth of EN76<sup>T</sup> was evaluated compared with a chemolithoautotrophic culture supplemented with 1 mM ammonium and 2 mM bicarbonate (default culture). Growth is scored as: ++, positive effect; +, similar to default culture; +/−, negative effect; −, total inhibition of growth. All incubations were performed at least in duplicate. The table is an extended version of Table S1 of Touna et al. (2011). The default ammonium concentration added to cultures was 1 mM (if not stated otherwise) and the default pyruvate concentration added to cultures was 0.5 mM (if not stated otherwise).

<table>
<thead>
<tr>
<th>Substrate added</th>
<th>Substrate</th>
<th>Growth</th>
</tr>
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<tbody>
<tr>
<td>Ammonium</td>
<td>Pyruvate</td>
<td></td>
</tr>
<tr>
<td>Bicarbonate (&gt;2 mM (up to 10 mM))</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Organic acids (TCA cycle)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate (0.05–2 mM)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Citrate, succinate, fumarate, malate (0.1–0.5 mM)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Pyruvate (0.05–10 mM)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Pyruvate (0.5 mM)</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Oxaloacetate (0.5 mM)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>α-Ketoglutarate (0.5 mM)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Glyoxylate (0.5 mM)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Sugars and sugar alcohols</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, fructose, lactose, arabinose, sucrose, galactose, mannose (0.5–1 mM)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Ribose (0.5 mM)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Glycerol (0.1 %, v/v)</td>
<td>Yes</td>
<td>No</td>
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<tr>
<td>Complex organic compounds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptone, yeast extract (0.05 %, w/v)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Nitrogen compounds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5–3 mM</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>0.5–15 mM</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Urea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5–1 mM</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Amino acids</td>
<td></td>
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</tr>
<tr>
<td>l-Alanine, d-alanine, l-glutamine, l-aspartic acid (0.1 g l&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>l-Glutamic acid, d-glutamic acid (0.1 g l&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Amino acid mixture (1 mM), Casamino acids (0.05 %, w/v)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Amines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trimethylamine, ethanolamine (1 mM)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Methanolamine (1 mM)</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Methylamine (0.5 mM)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Nitrate (1 mM)</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Taurine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25–0.5 mM</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>0.25–0.5 mM</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Nucleobases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrimidine, purine (0.1–1 mM)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Cytidine (0.1–1 mM)</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

DNA isolation and phylogenetic analyses. Isolation of DNA from the enrichment culture of strain EN76<sup>T</sup> and 454 pyrosequencing on a 454/FLX-Titanium sequencer (Roche) have been described in detail previously (Touna et al., 2011). The genome sequence was assembled and annotated on the MicroScope platform (Vallenet et al., 2006, 2009). The 16S rRNA, amoA and amoB gene sequences of EN76<sup>T</sup> have been deposited previously in GenBank under accession numbers FR773157, FR773159 and FR773160, respectively (Tourna et al., 2011). Phylogeny reconstruction of archaeal 16S rRNA genes was based on the alignment of 1202 bp gene fragments with four independent methods: CLUSTAL W (Thompson et al., 1994), MUSCLE (Edgar, 2004), MAFFT (Katoh et al., 2005; Katoh & Toh, 2010), and T-Coffee (Di Tommaso et al., 2011). The final consensus multiple sequence alignment of the four methods was calculated with MergeAlign (Collingridge & Kelly, 2012). After manual curation, hypervariable positions that could not be aligned unambiguously were excluded. Maximum-likelihood phylogenetic trees and bootstrap support values were calculated with RaxML VI-HPC (Stamatakis, 2006; Stamatakis et al., 2008) based on the GTR + I + G.
spread energy (and perhaps also carbon) source for AOA
marine sediments, indicating that urea is a more wide-

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'sosphaera gargensis' Ga9.2 (both associated with group

Storage. Cells of strain EN76T were harvested, suspended in 40 %
(v/v) glycerol and stored at −80 °C. Growth could be restored after
preservation for 12 months by carefully thawing cells on ice and
removing the glycerol by centrifugation of cells prior to inoculation
into fresh medium.

Results and Discussion

Metabolism

Strain EN76T is a mesophilic and neutrophilic organism, growing at 28–47 °C and pH 6–8.5 (Touna et al., 2011). It
produces energy by oxidizing ammonia aerobically to
nitrite (Fig. 1a). Strain EN76T grows equally well on urea as
an energy source, with production of about 2 mmol nitrite
per mol urea (Touna et al., 2011). In contrast to ‘Candidatus Nitrosopumilus maritimus’ SCM1 (group I.1a),
strain EN76T and its close relative ‘Candidatus Nitroso-
sphaera gargensis’ Ga9.2 (both associated with group
I.1b) possess genes encoding urease and urea transporters
(Spang et al., 2012; Stieglmeier et al., 2014a; Touna et al.,
2011; Walker et al., 2010) and, accordingly, growth on urea
has also been demonstrated in enrichment cultures of
‘Candidatus Nitrososphaera gargensis’ Ga9.2 (Spang et al.,
2012). Genes for urea utilization were recently also found
in metagenomic analyses of marine group I thaumarch-
aeotes from Arctic and meso-/bathypelagic waters and
marine sediments, indicating that urea is a more wide-

spread energy (and perhaps also carbon) source for AOA
(Alonso-Sáez et al., 2012; Park et al., 2012) than was
previously appreciated. The NO scavenger carboxy-PTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-
oxyl-3-oxide] (Amano & Noda, 1995) has been shown to
inhibit growth and nitrite production of both strain
EN76T (Shen et al., 2013) and ‘Candidatus Nitroso-
pumilus maritimus’ SCM1 (Yan et al., 2012), indicating
an important role for NO in their energy metabolism.
In addition, hydroxylamine is probably an intermediate
during the oxidation of ammonia to nitrite, as shown
for AOB [Arp & Stein (2003) and references therein;
Hooper & Terry (1979)], and recently also shown for the
marine AOA ‘Candidatus Nitrosopumilus maritimus’
SCM1 (Vajrala et al., 2013). Strain EN76T tolerated am-

monium concentrations up to 15 mM and nitrite con-

centrations up to 10 mM, as reported previously (Touna
et al., 2011). Thus, EN76T is less sensitive to high ammno-

ium and nitrite concentrations than, for example, its
close relative ‘Candidatus Nitrososphaera gargensis’ Ga9.2
and the marine strain ‘Candidatus Nitrosopumilus mari-
timus’ SCM1 (Hatzenpichler et al., 2008; Körnke et al.,
2005). The strain produces nitrous oxide (N₂O) in
amounts comparable to those of AOB under oxic condi-
tions (4.6 ± 0.6 amol N₂O cell⁻¹ h⁻¹). However, in con-
trast to AOB, N₂O production does not increase under
reduced oxygen levels, and might occur via a hybrid
formation mechanism (Stieglmeier et al., 2014b).

Strain EN76T is a mixotrophic organism that requires
organic acids (e.g. pyruvate, oxaloacetate, α-ketoglutarate
or glyoxylate) to stimulate growth (Table 1; Touna et al.,
2011). Other organic substrates, such as sugars or amines,
did not have a positive effect on growth of EN76T (Table 1). Growth stimulation by organic acids has recently also been reported for the marine strain ‘Candidatus Nitroso- 
pumilus maritimus’ SCM1 (Stahl & de la Torre, 2012; Urakawa et al., 2011). Comparative genomic analyses of strain EN76T, ‘Candidatus Nitroso- 
pumilus maritimus’ SCM1 (Walker et al., 2010) and ‘Candidatus Nitroso-
spheara gargensis’ Ga9.2 (Spang et al., 2012) revealed the presence of genes encoding transporters for amino acids, sulfonates (e.g. taurine) and glycerol in all three strains (P. Offre, M. Kerou, A. Spang and C. Schleper, unpublished). In addition, EN76T encodes putative nucleobase transporters. Therefore, different amino acids, taurine, glycerol and nucleobases were tested in various concentrations as possible substrates for EN76T (Table 1). However, none of the above-mentioned compounds had a positive effect on growth under the conditions tested. Instead, glycerol, several amino acids and nucleobases inhibited growth.

Strain EN76T was initially described to grow optimally in FWM supplemented with 1 mM pyruvate and 1 mM NH4Cl at 37 °C and pH 7.5 (Tourna et al., 2011), with a doubling time of approximately 45 h (based on cell counts). However, the generation time of strain EN76T decreased progressively from 45 to 27.5 h during continuous cultivation and growth optimizations over 3 years (Fig. 1b). In order to reassess the optimal growth conditions and possible interactions between the three known factors that influence growth rate (i.e. temperature and ammonium and pyruvate concentrations) comprehensively, we used a DoE screening and optimization strategy (Box & Draper, 1987; Box & Lucas, 1959; Rittmann & Herwig, 2012). As an example for the output of those experiments, the RSM shown in Fig. S1 illustrates the effect of temperature and pyruvate concentration on the maximum specific growth rate ($\mu_{\text{max}}$) at a constant ammonium concentration of 2.5 mM. Based on the DoE data and the calculated model obtained after varying temperature, ammonium and pyruvate concentrations, we predicted the optimal growth conditions of EN76T (see Table S2). The highest maximum specific growth rate ($\mu_{\text{max}}$ = 0.024 h$^{-1}$) should be reached at 41.83 °C, 1.05 mM pyruvate and 2.59 mM NH4Cl (with a desirability of 84%). This corresponds to a generation time of 29.0 h. In order to verify $\mu_{\text{max}}$ and the generation time predicted by the RSM experimentally, we grew the strain at 42 °C, 1 mM pyruvate and 2 mM NH4Cl and obtained a maximum specific growth rate ($\mu_{\text{max}}$ = 0.023 h$^{-1}$). In addition, this generation time and a generation time of 30.1 ± 0.6 h (based on nitrite production). Similar values for growth rate and generation time were obtained using cell counts of EN76T for the calculation ($\mu_{\text{max}}$ = 0.026 h$^{-1}$; generation time 27.5 ± 6.5 h). These experimentally determined values are close to the generation time (29.2 h) and a maximum specific growth rate ($\mu_{\text{max}}$ = 0.024 h$^{-1}$) predicted by the model equation under the tested conditions (Table S2). The model equation was additionally verified by recalculating the generation time reported by Tourna et al. (2011). The generation time based on the previously used growth conditions was calculated as 42.0 ± 2.7 h, which is close to the initial experimental determination of 45 h (Tourna et al., 2011). Enrichment cultures of the closely related strain ‘Candidatus Nitrososphaera gargensis’ Ga9.2 have been reported to grow at 46 °C with an ammonium concentration of 1 mM (Hatzenpichler et al., 2008).

Given that thaumarchaeotes have been shown to be light-
sensitive (French et al., 2012; Merbt et al., 2012), strain EN76T was incubated in the dark. When cultivated in larger volumes (>100 ml), cultures were shaken at 150 r.p.m. Although the strain grows aerobically [21% (v/v) O2 in the gas phase], it can grow at oxygen concentrations as low as 3% (v/v) O2 in the gas phase (Stieglmeier et al., 2014b).

Tests of various rubber stoppers indicated that growth of EN76T was inhibited completely by black butyl and red isoprene rubber stoppers, although it tolerated grey and blue butyl rubber stoppers, as well as grey natural rubber stoppers (Table S1). Inhibition of the activity of methan- 

trophic bacteria by black butyl rubber stoppers has been reported previously (Ettwig et al., 2009).

EN76T is not affected by water-soluble antibiotics like kanamycin, streptomycin, carbenicillin, ampicillin (Tourna et al., 2011) and ofloxacin, but is inhibited by antibiotics that are soluble in ethanol or DMSO (e.g. chlorampheni-

col). Recently, the effects of nitrification inhibitors (e.g. nitrapyrin, allylthiourea and dicyandiamide) and the anti-
biotic sulfathiazole, which are commonly used in agricult-

ture and livestock production, on the AOA strain EN76T and a strain of the ammonia-oxidizing bacterium Nitro-
sospora multiformis have been tested (Shen et al., 2013). Nitrapyrin had a stronger inhibitory effect on EN76T compared with the bacterium, whereas dicyandiamide, the copper chelators allylthiourea and amidinothiourea and the antibiotic sulfathiazole had a weaker inhibitory effect on EN76T (Shen et al., 2013).

**Morphology**

The irregular cocccoid cells of strain EN76T had a diameter of 0.78 ± 0.13 μm (n=16) and usually occurred as single cells, although clusters comprising several cells were sometimes observed. Cells were motile and possess archaella (archaeal flagella that are homologous to type IV pili; Jarrell & Albers, 2012) with a diameter of 12.0 ± 1.8 nm (Fig. 2g, h). Genes encoding *Crenarchaeota*-like type-2 flagellins and *Euryarchaeota*-like chemotaxis proteins (Fig. S2) were found in the genome of EN76T, supporting the conclusion that EN76T is probably motile. *Crenarchaeota*-like type-2 fla gene clusters have been found in ‘*Candidatus Nitrososphaera gargensis*’ Ga9.2 and the group I.1a-related strain ‘*Candidatus Nitrosoarchaeum limnia*’ SPB1, but not in the genome of ‘*Candidatus Nitrosopumilus maritimus*’ SCM1 (Blainey et al., 2011; Spang et al., 2012; Walker et al., 2010). In addition, pili with a diameter of 6.4 ± 1.3 nm were observed (not shown). The diameters of both appendages are within the size ranges described for other archaea (Klingl et al., 2013).
In order to investigate the ultrastructure of EN76\textsuperscript{T}, ultrathin sections of chemically fixed and embedded cells were prepared (Fig. 2c–f). Cells often contained one or two electron-dense particles with a size of $87 \pm 15$ nm (Fig. 2f). Similar electron-dense particles present in anaerobic ammonium-oxidizing (anammox) bacteria have been shown to be enriched in iron and were proposed to constitute bacterioferritins, which are known as iron storage proteins (Andrews et al., 2003). Furthermore, the genome of EN76\textsuperscript{T} carries genes for proteins belonging to the ferritin/Dps domain proteins (Nvie_002890, Nvie_017250, Nvie_020180, Nvie_028750, Nvie_029390), which have also been identified in genomes of other members of the Thaumarchaeota (Spang et al., 2012). Dps proteins are known to protect cells against oxidative stress by binding iron (Haikarainen & Papageorgiou, 2010). These findings suggest that the electron-dense particles present in strain EN76\textsuperscript{T} may have a role in iron storage and cellular protection. Tubule-like structures were also identified in cells of strain EN76\textsuperscript{T} (Fig. 2f), and may have a cytoskeletal function, as proposed previously for the hexagonal long tubule-like structures found inside the anammoxosome of anammox bacteria (Lindsay et al., 2001; van Niftrik & Jetten, 2012). Although genomes of members of the Thaumarchaeota encode an FtsZ homologue, it is unlikely that the tubule-like structures are formed by this protein, because the marine strain ‘Candidatus Nitrosopumilus maritimus’ SCM1 was shown to recruit the Cdv mechanism primarily during cell division (Pelve et al., 2011). Small inclusions of less electron-dense material (Fig. 2d) compared with the cytoplasm were observed in cells of strain EN76\textsuperscript{T}, which might be polyphosphate or glycogen storage granules, for example (Klingl et al., 2013). Strain EN76\textsuperscript{T} possesses a clearly discernible area within the cytoplasm (Fig. 2d, e). So far, we could not identify a lipid or proteinaceous layer surrounding this area, which is characteristic for intracellular microcompartments found in bacteria. Large intracellular compartments, such as the carboxysome and anammoxosome, have been described in several bacteria (Erbilgin et al., 2014; Shively et al., 1973; van Niftrik et al., 2004). However, compartmentalization has been reported in only few archaea, e.g. a two-membrane system in the crenarchaeotal genus Ignicoccus (Huber et al., 2000) and in the euryarchaeote Methanomassiliicoccus luminyensis (Dridi et al., 2012). Larger intracellular particles like polyhydroxyalkanoate granules have been found in halophilic archaea (Fernandez-Castillo et al., 1986). Raman spectroscopy analyses have indeed shown that strain EN76\textsuperscript{T}
S-layer proteins are one, or even the only, cell-wall component of several archaea and bacteria (Rachel et al., 1997). The S-layer usually consists of a single type of protein arranged in a regular lattice pattern, probably driven by entropic processes (Eichler, 2003; Sleytr et al., 2001, 2007). Depending on the arrangement, these pseudocrystalline areas depict highly ordered opaque p1- or p2-symmetry, square p4-symmetry or sixfold p3- or p6-symmetry. TEM analyses of both freeze-etching replicas (Fig. 3a) and negatively stained S-layer sheets (Fig. 3b) of cells of strain EN76T showed a regular pattern of a two-dimensional protein crystal with 6-fold symmetry. By further analysing the images with ANIMETRA CRYSTALS, distinction between p3- and p6-symmetry could be achieved. Correlation averaging of electron micrographs of both freeze-etched cells (Fig. 4a) and purified S-layer sheets (Fig. 4c) revealed an unexpected p3-symmetry of the S-layer protein. The relief illustration of the image in Fig. 4(a), shown in Fig. 4(b), revealed that the unit cell of the S-layer consists of a trimer of protein trimers. Additionally, the triangular shape of the pores between the protein trimers can be seen, which could function as a molecular sieve, separating the surrounding medium from the pseudo-periplasm, located between the S-layer and the cytoplasmic membrane (reviewed by Sleytr et al., 1993). Determination of the lattice constants after correlation averaging yielded slightly differing values for the two preparation methods, with 21.1 nm for freeze-etching and 20.2 nm for negative staining. This could be caused by the preparation itself, by calculation errors or by the initial choice of the reference area for correlation averaging. The range of lattice values obtained here is, nevertheless, in the same range as those for other p3-symmetry S-layers reported for members of the Sulfolobales, which are all around 21 nm (König et al., 2007; Veith et al., 2009). Up to now, p3-symmetry was thought to be unique to the order Sulfolobales, given that all investigated species from this group had this symmetry and a consistent lattice value (Baumeister & Lembcke, 1992; Deatherage et al., 1983; Grogan, 1996; Klingl et al., 2013; König et al., 2007; Lembcke et al., 1991, 1993; Prüschenk & Baumeister, 1987; Prüschenk et al., 1987; Taylor et al., 1982; Veith et al., 2009). Thus, the occurrence of p3-symmetry in the thaumarchaeote EN76T excludes this characteristic as a taxonomic marker for the order Sulfolobales (Klingl et al., 2011; König et al., 2007).

As described previously (Sinninghe Damsté et al., 2012), the intact polar lipids of cells of strain EN76T consist of GDGTs bound to the polar head groups monohexose, dihexose, trihexose, phosphohexose or hexose-phosphohexose. Crenarchaeol and its regioisomer, both GDGTs with one cyclohexane and four cyclopentane rings (Sinninghe Damsté et al., 2002), were the major core lipids (80%; Sinninghe Damsté et al., 2012). So far, crenarchaeol has been found exclusively in members of the Thaumarchaeota.
Phylogenetic analyses

Initial phylogenetic analyses of 16S RNA gene sequences from environmental studies placed the 'mesophilic archaea' (i.e. *Thaumarchaeota*), closely related to strain EN76<sup>T</sup>, as a deep-branching group of the *Crenarchaeota* (DeLong, 1992; Fuhrman et al., 1992). However, later analyses based on full rRNA gene sequences, concatenated ribosomal protein sequences and full-genome sequence comparisons provided strong evidence that these mesophilic and aerobic AOAs represent a distinct phylum, the *Thaumarchaeota* (Brochier-Armanet et al., 2008; Spang et al., 2010). Members of this phylum encode a specific set of information-processing genes that is distinct from those in the phyla *Crenarchaeota* and *Euryarchaeota*, as well as in the proposed phylum *Korarchaeota* (Brochier-Armanet et al., 2008; Spang et al., 2010) and in other currently proposed phyla (Brochier-Armanet et al., 2011; Rinke et al., 2013; Spang et al., 2013). Strain EN76<sup>T</sup> is affiliated with group I.1b of the *Thaumarchaeota* (also known as the 'soil group') based on 16S rRNA gene phylogeny (Fig. 5), also showing a consistent phylogenetic clustering based on concatenated AmoAB protein sequences (Tourna et al., 2011). *Candidatus Nitrososphaera garrensis* Ga9.2 shares 97% 16S rRNA gene sequence identity with strain EN76<sup>T</sup> (Tourna et al., 2011). *Candidatus Nitrosopumilus maritimus* SCM1 is currently the only described pure culture of the second major group within group I.1a of the *Thaumarchaeota* (Könneke et al., 2005; Walker et al., 2010), and shares 85% 16S rRNA gene sequence identity with strain EN76<sup>T</sup>. However, the name 'Nitrosopumilus maritimus' has not been validly published and does not have standing in nomenclature. Based on 16S rRNA gene sequence identity, *Thermofilum pendens* Hrk 5 (81% 16S rRNA gene sequence identity) and *Methanothermus fervidus* DSM 2088<sup>T</sup> (79%) represent the closest related cultivated strains of species with validly published names from the phyla *Crenarchaeota* and *Euryarchaeota*, respectively (Fig. 5). Strain EN76<sup>T</sup> has a DNA base composition of 52.7 mol% G+C (Tourna et al., 2011), which is similar to that of *Candidatus Nitrososphaera garrensis* Ga9.2 (48.4 mol%) and higher than that of group I.1a strains such as *Candidatus Nitrososphaera garrensis* SCM1 (34.2 mol%), *Candidatus Nitrosoarchaeum koreensis* MY1 (32.7 mol%) and *Candidatus Nitrosoarchaeum limnia* SFB1 (32.4 mol%) (Blaine et al., 2011; Kim et al., 2011; Rinke et al., 2012; Walker et al., 2010). In conclusion, groups I.1a and I.1b differ greatly in their G+C content and form two highly supported distinct phylogenetic lineages based on both 16S rRNA and amoA gene sequences (Fig. 5).

Description of *Nitrososphaera viennensis* sp. nov.

*Nitrososphaera viennensis* (vi.en.nen’sis. N.L. fem. adj. viennensis from Vienna, where the type strain was isolated and characterized).

Irregular cocci with a diameter of 0.78 ± 0.13 µm. Occur as single cells and as clusters of several cells. Cells exhibit archaella (12.0 ± 1.8 nm) and archaeal pili (6.4 ± 1.3 nm) as cell appendages, and clearly discernible areas of high and low electron density and tubule-like structures in the cytoplasm. Cells possess an S-layer with p3-symmetry. Grows at pH 6–8.5, with an optimum at pH 7.5. The temperature optimum is 42 °C; grows at 28–47 °C. Energy is produced by oxidizing ammonia to nitrite with oxygen as electron acceptor. Optimal NH<sub>4</sub>Cl concentration for growth is 2.6 mM, but concentrations up to 15 mM are tolerated. Nitrite concentrations up to 10 mM are tolerated. Urea can be used as substrate. N<sub>2</sub>O is formed as a side product during ammonia oxidation. Mixotrophic growth is observed with bicarbonate and small carboxylic acids, i.e. pyruvate, α-ketoglutarate, oxaloacetate or glyoxylate, as carbon sources. The following substrates have a negative effect or inhibit growth under the conditions tested: ribose, glycerol, peptone, yeast extract, L-alanine, D-alanine, L-glutamate, L-aspartic acid, an amino acid mixture, Casamino acids, methyleamine, trimethylamine, ethanalamine, methanolamine, nitrate, pyrimidine and purine.

The type strain, EN76<sup>T</sup> (= DSM 26422<sup>T</sup> = JCM 19564<sup>T</sup>), was isolated from a garden soil in Vienna, Austria. The DNA base composition of the type strain is 52.7 mol% G+C.

Description of *Nitrososphaeraceae fam. nov.*

*Nitrososphaeraceae* (Niтро.so.sphaе.ra’ce.ae. N.L. fem. n. *Nitrososphaera* type genus of the family; L. suff. -aceae ending to denote a family; N.L. fem. pl. n. *Nitrososphaeraceae* the family of the genus *Nitrososphaera*).

The description is the same as for the genus *Nitrososphaera*. The type genus is *Nitrososphaera*.

Description of *Nitrososphaerales ord. nov.*

*Nitrososphaerales* (Niтро.so.sphaе.ра’les. N.L. fem. n. *Nitrososphaera* type genus of the order; N.L. suff. -ales ending to denote an order, N.L. fem. pl. n. *Nitrososphaerales* the order of the genus *Nitrososphaera*).

The name *Nitrososphaerales* refers to the former group I.1b (or 'soil group') within the phylum *Thaumarchaeota*. Cultivated organisms of this order have an irregular coccoid cell shape and occur predominantly in terrestrial ecosystems. By contrast, cells of all known organisms affiliated with group I.1a (order 'Nitrosopumilales') are rod-shaped. The order *Nitrososphaerales* comprises a highly supported distinct
phylogenetic group based on 16S rRNA gene phylogeny (Fig. 5). The 16S rRNA genes of all members share ≥ 90 % sequence identity, as do all members of other phylogenetically well-defined groups, e.g. the tentative orders 'Nitrosopumilales' (represented by 'Candidatus Nitrosopumilus maritimus' SCM1; Könneke et al., 2005), 'Nitrosotaleales' (represented by 'Candidatus Nitrosotalea devanaterra' Nd1; Lehtovirta-Morley et al., 2011) and 'Nitrosocaldales' (represented by 'Candidatus Nitrosocaldus yellowstonii' HL72; de la Torre et al., 2008). The names of these orders are currently not validly published, given the lack of representative organisms in pure culture, or depositions in culture collections. The type genus is Nitrososphaera.

**Description of Nitrososphaeria classis nov.**

Nitrososphaeria (Ni.tro.so.sphaer.i.a. N.L. fem. n. *Nitrososphaera* the type genus of the type order of the
class; N.L. suff. -ia ending to denote a class, N.L. neut. pl. n. *Nitrososphaeria* the class of the order *Nitrososphaerales*).

Cultivated strains within this class possess genes of both FtsZ- and Cdv-based cell division systems and have a topoisoenzyme IB. Similar to euryarchaeal strains, but in contrast to crenarchaeal strains, they have DNA polymerases B and D, eukaryote-like histones (H3/H4) and only one copy of the proliferating cell nuclear antigen and lack genes for RNA polymerase G (Brochier-Armanet et al., 2011; Spang et al., 2010). Crenarchaeol is the major core lipid and is not known to occur in any other bacterial or archaeal phylum (Pitcher et al., 2010; Schouten et al., 2008; Sinninghe Damsté et al., 2002, 2012). Additionally, genes encoding an ammonia monooxygenase have been found exclusively in all lineages within the class, among all archaeal taxa described, and might therefore be considered a distinctive feature. So far, all investigated genomes of members of this class contain genes encoding key enzymes of the 3-hydroxypropionate/4-hydroxybutyrate pathway, including acetyl-CoA carboxylase, 4-hydroxybutyl-CoA dehydratase and methylmalonyl-CoA mutase, suggesting that members of the phylum *Thaumarchaeota* might assimilate their cellular carbon via a modified version of this pathway (Berg et al., 2007; Blaine et al., 2011; Kim et al., 2011; Mosier et al., 2012a, b; Park et al., 2012; Spang et al., 2012; Walker et al., 2010). The class comprises a highly supported monophyletic lineage in the 16S rRNA gene phylogeny of the *Archaea* (Fig. 5). The type order is *Nitrososphaerales*.

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