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A hydrophobic ammonia-oxidizing archaeon of the *Nitrosocosmicus* clade isolated from coal tar-contaminated sediment

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Summary

A wide diversity of ammonia-oxidizing archaea (AOA) within the phylum *Thaumarchaeota* exists and plays a key role in the *N* cycle in a variety of habitats. In this study, we isolated and characterized an ammonia-oxidizing archaeon, strain MY3, from a coal tar-contaminated sediment. Phylogenetically, strain MY3 falls in clade '*Nitrosocosmicus*' of the thaumarchaeotal group I.1b. The cells of strain MY3 are large 'walnut-like' cocci, divide by binary fission along a central cingulum, and form aggregates. Strain MY3 is mesophilic and neutrophilic. An assay of ^{13}C-bicarbonate incorporation into archaeal membrane lipids indicated that strain MY3 is capable of autotrophy. In contrast to some other AOA, TCA cycle intermediates, i.e. pruvate, oxaloacetate and α-ketoglutarate, did not affect the growth rates and yields of strain MY3. The attachment of cells of strain MY3 to XAD-7 hydrophobic beads and to the adsorbent vermiculite demonstrated the potential of strain MY3 to form biofilms. The cell surface was confirmed to be hydrophobic by the extraction of strain MY3 from an aqueous medium with *p*-xylene. Our finding of a strong potential for surface attachment by strain MY3 may reflect an adaptation to the selective pressures in hydrophobic terrestrial environments.

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Introduction

Nitrification is a key biogeochemical process in which ammonia is converted, stepwise, to nitrite and then to nitrate. Recently, individual cultures of bacteria have been discovered that are capable of completing both nitrification steps intracellularly (Daims *et al.*, 2015; van Kessel *et al.*, 2015); the ecological relevance of this new 'comammox' process has yet to be explored. In contrast, the traditional paradigm of nitrification's two steps being mediated by independent yet cooperating microbial populations, i.e. the first step mediated by ammonia-oxidizing microorganisms (*Bacteria* and *Archaea*) and the second by nitrite-oxidizing bacteria (NOB), has been shown to be ecologically important for many years (Prosser and Nicol, 2012). Remarkably, however, the environmental factors determining distribution and diversity of populations that initiate nitrification by oxidizing ammonia are still not clearly resolved. It has been established that the ammonia-oxidizing archaea (AOA) of the phylum *Thaumarchaeota* can be numerically dominant and thus are likely active in various terrestrial environments (Prosser and Nicol, 2012). The AOA in soils and sediments represent great phylogenetic diversity, and the differences in nitrification rates among different soils are attributed to distinct populations of AOA with specific nitrification characteristics (Prosser and Nicol, 2012). Adaptations to physical-chemical conditions (e.g., pH, moisture, salinity, temperature, *N* concentration and organic carbon content, among others) have been proposed (Prosser and Nicol, 2012) to explain the niche differentiation among the diverse AOA taxonomic clades.

The ecophysiological properties of AOA are routinely investigated using pure and/or enrichment culture strategies, which provide information on the relationships

among AOA clades and on the ecological niches that are inhabited. Representatives of many diverse clades of terrestrial AOA have been characterized (Hatzenpichler *et al*., 2008; de la Torre *et al*., 2008; Jung *et al*., 2011; Lehtovirta-Morley *et al*., 2011; Kim *et al*., 2012; Lebedeva *et al*., 2013; Stieglmeier *et al*., 2014; Jung *et al*., 2014a; Lehtovirta-Morley *et al*., 2016). The mode of carbon metabolism across the AOA clades is a characteristic that provides insight in defining the ecological roles of various AOA taxa. For example, mixotrophic metabolism is proposed to be a key trait of some soil (Tourna *et al*., 2011) and marine (Qin *et al*., 2014) AOA clades, based on an obligatory requirement of organic acids for growth. By contrast, the addition of organic carbon substrates had no influence on and/or was not essential for the growth of other isolated AOA (Könneke *et al*., 2005; French *et al*., 2012; Lehtovirta-Morley *et al*., 2014; Jung *et al*., 2014a; Palatinszky *et al*., 2015; Lehtovirta-Morley *et al*., 2016). Thus, the identification of carbon-assimilation phenotypes associated with particular AOA clades is valuable in the prediction of metabolic activity, particularly nitrification, of AOA.

There are still many novel terrestrial clades of AOA that defy cultivation. A clade affiliated with fosmid clone 29i4 was initially identified during the screening of soil archaeal fosmid clones (Quaiser *et al*., 2002). The members of this clade are widely observed in terrestrial habitats that include the rhizosphere (Simon *et al*., 2000; 2005; Xu *et al*., 2012), paddy soils (Xia *et al*., 2011; Wang *et al*., 2015), acidic soil (Wu and Conrad, 2014), arctic soil (Alves *et al*., 2013), municipal wastewater treatment plants (Sonthiphand and Limpiyakorn, 2011; Sauder *et al*., 2012) and fuel oil-affected environments (Kasai *et al*., 2005; Mußmann *et al*., 2011) by analysis of 16S rRNA and *amoA* gene sequences. Very recently, an archaeal strain, *Nitrosocosmicus franklandus* C13, of this clade with high tolerance to ammonia concentration was isolated from an arable soil (Lehtovirta-Morley *et al*., 2016). To provide further insights into the ecological niche and diversity AOA of the '*Nitrosocosmicus*' clade within the *Thaumarchaeota*, this study describes the ecophysiological properties of a terrestrial ammonia-oxidizing archaeon, *Nitrosocosmicus* sp. strain MY3, which was isolated from coal tar-contaminated sediment.

Results and discussion

Isolation of an ammonia-oxidizing archaeon, strain MY3

An ammonia-oxidizing archaeal enrichment culture was obtained from surface sediment rich in decaying forest-leaf litter and contaminated by hydrocarbons characteristic of coal-tar waste (see the Supporting Information). The hydrocarbon-rich surface sediment sample was collected from a low lying seep area in which groundwater from a buried coal tar site emerges from a hillside and flows into a small freshwater stream (Madsen *et al*., 1991; Jeon *et al*., 2003). After the ammonia-oxidizing culture was repeatedly transferred (10% v/v) to fresh artificial freshwater medium (AFM) the archaeal cell-dominant culture was serially diluted to extinction in 10-fold steps using 96-well plates with deep wells, and the highest dilutions with nitrifying activity were selected. Among the dilutions, one that contained a clonal and pure culture of an ammonia-oxidizing archaeon designated strain MY3 was selected for further study. During exponential growth of strain MY3 approximate stoichiometric oxidation of ammonia to nitrite was observed (Supporting Information Fig. S1). The maximum specific growth rate (μ_{max}) of strain MY3 is lower than other AOA including *N. franklandus* but was comparable with marine strain PS0 and acidic soil strain ND1 (Supporting Information Table S1). Urea was also utilized as an energy source consistent with the presence of a set of urea utilization genes (Supporting Information Fig. S2).

Strain MY3 grew in a wide range of pH values, 5.5–8.5, with the optimum growth at pH of 6.5–7.0 (Supporting Information Fig. S3A). Growth rates decreased in alkaline (pH $>$ 7.0) and acidic conditions (pH $<$ 6.5), with the incomplete oxidation of 0.5 mM ammonia (Supporting Information Fig. S3A). Strain MY3 grew within the temperature range of 25–30°C, with the optimum growth at 30°C (Supporting Information Fig. S3B), which is lower than those of other AOA strains of the I.1b group, *N. franklandus*, *Nitrososphaera viennensis* and *Nitrososphaera gargensis* (40–46°C) (Hatzenpichler *et al*., 2008; Stieglmeier *et al*., 2014; Lehtovirta-Morley *et al*., 2016). Therefore, the organism can be considered to be neutrophilic and mesophilic. The maximum concentration of ammonia that strain MY3 completely oxidized was approximately 1.85 mM, although the oxidation of ammonia was not inhibited by concentrations up to 10 mM (Supporting Information Fig. S3C). At 50 mM, ammonia oxidation was significantly inhibited. The properties of strain MY3 are summarized in the Supporting Information Table S1 for comparison with other AOA strains.

Strain MY3 shared 98.5% and 99.1% 16S rRNA gene similarities with the fosmid clone 29i4, which was obtained during one of the first metagenomic investigations of soil thaumarchaeal group I.1b (Quaiser *et al*., 2002), and *N. franklandus*, from an arable soil (Lehtovirta-Morley *et al*., 2016) respectively (Fig. 1 and Supporting Information Fig. S4). The *amoA* gene similarity of strain MY3 with *N. franklandus* was 91.7%. The similarities of the 16S rRNA and *amoA* gene sequences between strain MY3 and other cultivated soil strains of thaumarchaeotal group I.1b, *N. viennensis*, *N. gargensis*,

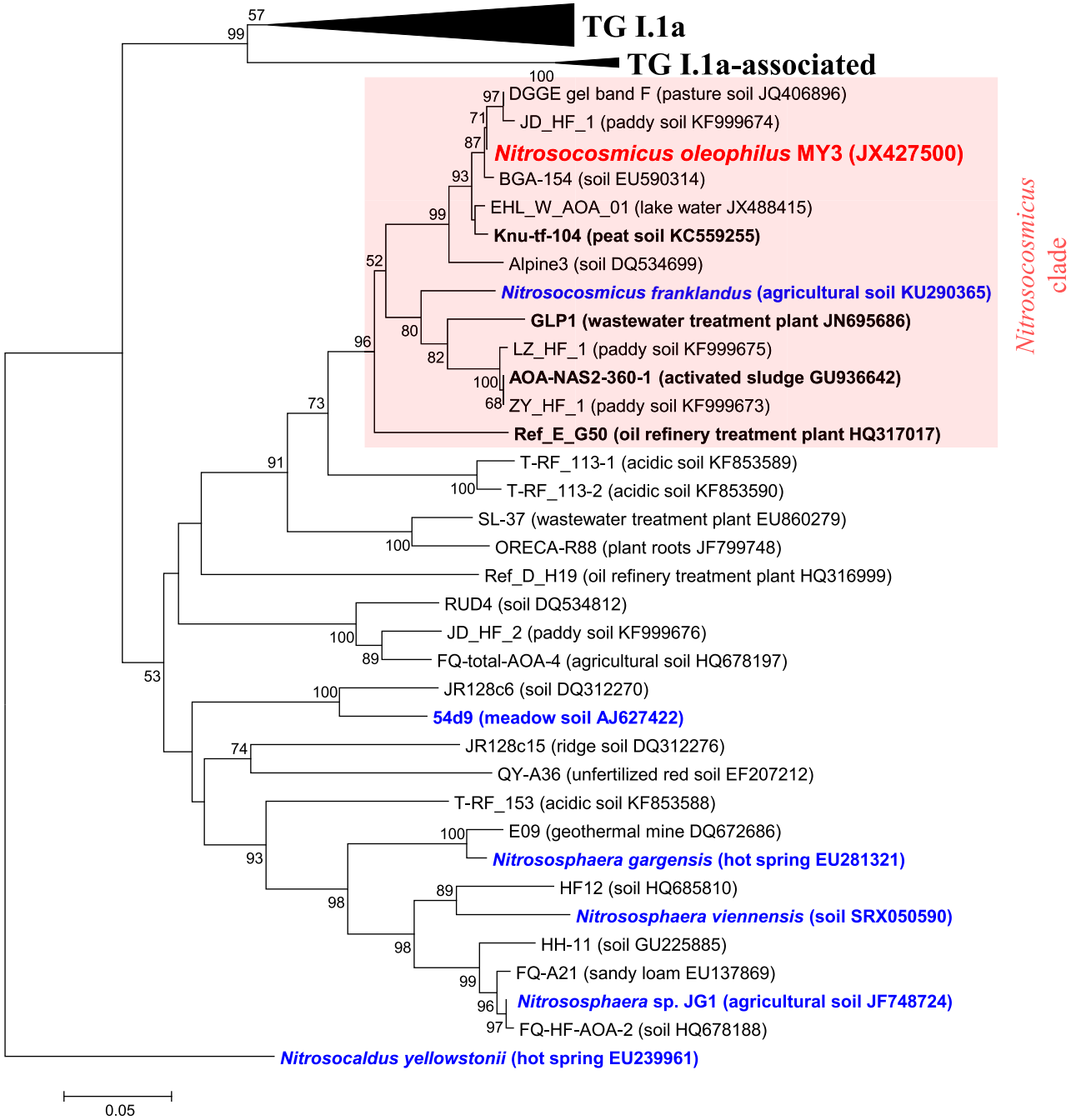

Fig. 1. Comparative phylogenetic analysis of *amoA* gene sequences from strain MY3. Branching patterns supported by bootstrap values (1 000 iterations) > 50%, according to the neighbour-joining method, are denoted by those bootstrap values. The strains marked in blue are reference strains or a fosmid clone of *Thaumarchaeota*, and the environmental sequences obtained from wastewater treatment plants are bold-faced. The putative '*Nitrosocosmicus*' clade is red-boxed. The origins of each clone or strain are indicated in parentheses with their accession numbers.

and strain JG1, were < 95.1% and < 76.1% respectively (Fig. 1 and Supporting Information Fig. S4).

The cells of strain MY3 were 'walnut-like' cocci with two combined hemispheres, and featured a central cingulum (septum) along which binary fission occurred (Fig. 2). The diameter of the cells was 1.1 μm ($n = 17$) (Fig. 2) and their volume was ca. 0.7 μm^3, which is 33-fold higher than that of *N. maritimus* (Martens-Habbena *et al.*, 2009), 19-fold higher than that of cells of the other strains of thaumarchaeotal group I.1a (Jung *et al.*, 2011; Qin *et al.*, 2014; Jung *et al.*, 2014a), and 2.8-fold higher than that of *N. viennensis* (Stieglmeier *et al.*, 2014) of

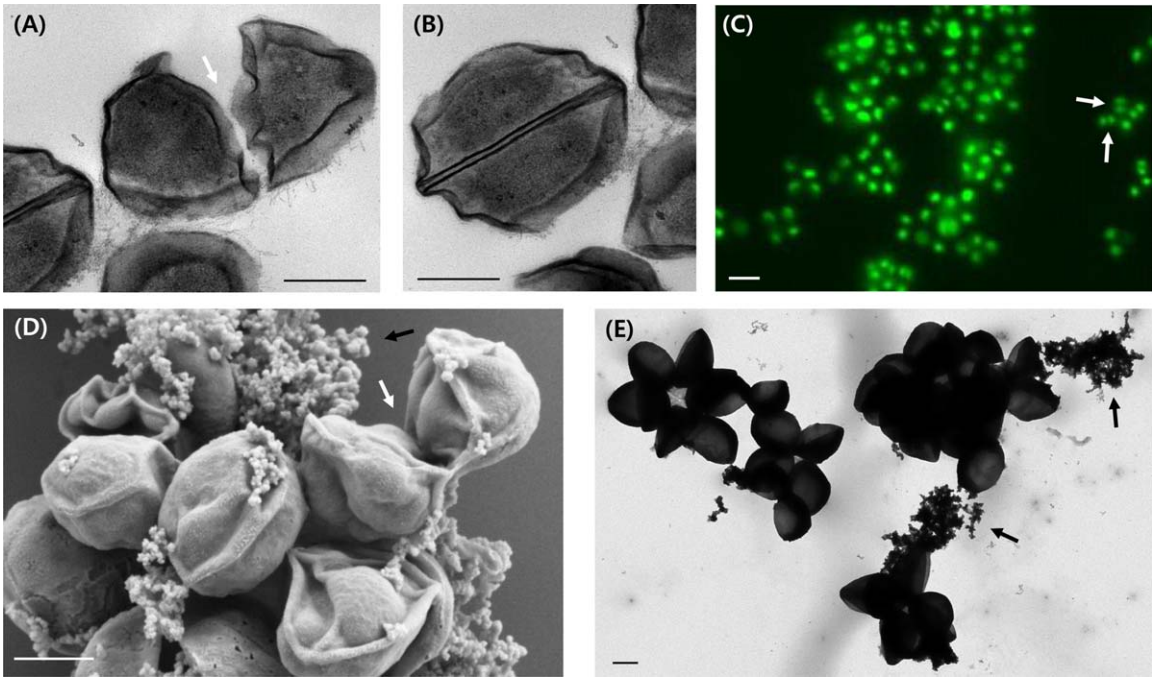

Fig. 2. Morphology of strain MY3.
A and B. Transmission electron micrograph (TEM) images of ultra-thin sections of cells of strain MY3 (scale bar: 0.5 μm).
C. Epifluorescence micrograph images of cells stained by DAPI (green) (scale bar: 2 μm).
D. Scanning electron micrograph image of strain MY3 cells (scale bar: 0.5 μm).
E. TEM image of cells of strain MY3 (scale bar: 0.5 μm). The white arrows in (A), (C) and (D) indicate dividing cells. The black arrows in (D) and (E) indicate putative EPS.

thaumarchaeotal group I.1b. The biomass of a single cell of strain MY3 was 108 ($\pm$ 4.7) fg protein cell^{-1}, which is the highest among AOA cells (10.2 and 24 fg protein cell^{-1} for *Nitrosopumilus maritimus* and *N. gargensis*, respectively) (Martens-Habbena *et al.*, 2009; Palatinszky *et al.*, 2015) and is comparable with that of AOB cells of a similar size (120 fg protein cell^{-1} of *Nitrosomonas europaea*) (Martens-Habbena *et al.*, 2009). This relatively large volume can explain both the high protein content per cell (see above) and the low final cell numbers of strain MY3 after consumption of an equivalent amount of ammonia (cell yield was lowest of almost all AOA studied, but similar to that of *N. franklandus* and AOB; Supporting Information Table S1).

Autotrophic growth

Carbon fixation by strain MY3 was investigated via a ^{13}C-carbon tracer experiment in which ammonia was oxidized in the presence of ^{13}C-labeled bicarbonate (5.0% ^{13}C-enriched). During the ^{13}C bicarbonate-amended growth experiments, these GDGTs became significantly enriched in ^{13}C. The ^{13}C content of the two predominant membrane lipids, i.e. GDGT-0 and crenarchaeol, was 4.6% and 4.8%, respectively, close to that of bicarbonate in the medium. Thus, bicarbonate was the sole carbon source for strain MY3 during growth. Mußmann and colleagues (2011) suggested that the members of thaumarchaeotal group I.1b from refinery wastewater treatment plants (closely related to strain MY3, with a 16S rRNA gene similarity of $>$99.3%) were not obligate chemolithoautotrophs, based on FISH-microautoradiography and GDGT tracer experiments, and the observation of low *in situ* transcription of archaeal *amoA*. Additionally, there is evidence suggesting members of the *Nitrosocosmicus* clade are heterotrophs or mixotrophs as their growth was enhanced by addition of organic substrates to media (Xu *et al.*, 2012). In contrast, *N. franklandus* (Lehtovirta-Morley *et al.*, 2016) and related populations from Arctic soil (Alves *et al.*, 2013) were found to be capable of growing under autotrophic conditions. Our GDGT tracer experiment demonstrated that strain MY3 has a chemolithoautotrophic life style. In good agreement, the MY3 genome (NCBI accession No. CP012850), as many other AOA genomes (Walker *et al.*, 2010; Kim *et al.*, 2011; Mosier *et al.*, 2012; Spang *et al.*, 2012; Jung *et al.*, 2014a), contained all key genes for 3-hydroxypropionate/4-hydroxybutyrate cycle (Supporting Information Table S2), which is responsible for autotrophic carbon fixation in AOA (Könneke *et al.*, 2014).

The effects of organic substrates on the growth of strain MY3 during ammonia oxidation are summarized in

Table 1. Effect of a range of supplemental organic substrates on the growth of strain MY3 in AFM. The default ammonia concentration added to the medium was 0.5 mM.

Substrate		Growth effect
Amino acid		
1 mM		
	Arg, His, Asp, Glu, Cys, Tyr, Trp	− −
	Lys, Ser, Gly, Pro, Met, Leu, Thr, Asn, Gln, Ala, Val, Ile, Phe	−
0.1 mM		
	Cys	− −
	Arg, His, Asp, Tyr, Lys, Ser, Met, Leu, Thr, Gln, Ala, Val, Glu, Phe, Asn, Gly, Pro, Ile, Trp	−
TCA cycle intermediate		
1 mM		
	Citrate, α-ketoglutarate, Oxaloacetate, Fumarate, Malate, Glyoxylate, Pyruvate, Succinate, Acetate	−
0.1 mM		
	Citrate, α-ketoglutarate, Malate, Oxaloacetate	−
	Fumarate, Glyoxylate, Pyruvate, Succinate, Acetate	NE
Saccharide (0.1 mM)		
	Sucrose, Lactose, Maltose, Trehalose, Cellobiose	NE
	Glucose, Fructose, Arabinose	+
	Mannose, Xylose, Galactose, Ribose	NE
Glycerol (0.1%, v/v)		−
Urea (0.5 mM)		NE
Alcohol (0.5 mM)		
	Ethanol	NE
	Methanol	−
Complex organic compounds		
100 mg l^{-1}		
	Peptone, Yeast extract, Casamino acid	+
10 mg l^{-1}		
	Peptone, Yeast extract, Casamino acid	+
Dipeptide (Biolog)[a]		−/−/NE

− −, Strong inhibition of ammonia oxidation rate.

−, Slight inhibition of ammonia oxidation rate.

NE, No Effect, similar to control culture.

+, Stimulation of ammonia oxidation rate.

a. Refer to Table S7.

Table 1. In the absence of ammonia, strain MY3 did not grow on any of the tested organic substrate, which indicated the absence of capability for strict heterotrophic growth. Unlike *N. viennensis* and the marine AOA strains, PS0 and HCA1, none of the tested organic acid (including pyruvate, oxaloacetate and α-ketoglutarate) significantly stimulated the growth of strain MY3 (Table 1 and Supporting Information Fig. S5). Recently α-keto acid requirement for AOA growth was interpreted as the scavenger function of α-keto acids to remove H_2O_2 generated during ammonia oxidation by AOA (Kim *et al.*, 2016). In good agreement with this, strain MY3 contains a putative catalase gene (NMY3_01750; see Supporting Information Fig. S6) in its genome, while most of cultivated AOA, except *N. gargensis* and *Nitrososphaera evergladensis*, lack this.

Despite the presence of putative genes for amino acid and peptide transporters in the MY3 genome (Supporting Information Table S3), amino acid or peptide substrate did not stimulate growth of strain MY3 (Table 1). Fructose, glucose and arabinose slightly increased the growth rates and yields of strain MY3 (Supporting Information Fig. S5), consistent with the presence of various putative genes for sugar transporters (Supporting Information Table S3). Despite of the presence of all of the genes for gluconeogenesis (Supporting Information Table S2), the absence of canonical key genes specific for glycolysis (glucosephosphate isomerase, phosphofructokinase and pyruvate kinase) implies that the sugars cannot be utilized as energy sources.

Biofilm formation

Strain MY3 was isolated from hydrophobic coal tar-contaminated terrestrial surface sediment. Interestingly, relatives of strain MY3 have frequently been reported as the dominant AOA in oil-affected environments (Kasai *et al.*, 2005; Mußmann *et al.*, 2011). Notably, the members of this clade are often reported to form aggregates and biofilms (Simon *et al.*, 2000; 2005; Weidler *et al.*,

2008; Mußmann *et al.*, 2011; Lehtovirta-Morley *et al.*, 2016). Strain MY3 cells also commonly aggregated to form clusters with putative extracellular polymeric substances (EPS) (Fig. 2). The hydrophobic nature and biofilm-forming potential of strain MY3 were investigated using three independent assays: (i) association with bacterial cells, (ii) attachment to both a hydrophobic bead (XAD-7) and a hydrated phyllosilicate mineral (vermiculite) and (iii) partitioning into *p*-xylene.

Actinobacterial strains were the dominant members of co-cultured bacteria during enrichment of strain MY3 (Jung *et al.*, 2014b); by contrast, *Actinobacteria* are not commonly observed in other AOA enrichment cultures (Lehtovirta-Morley *et al.*, 2011; Tourna *et al.*, 2011; Jung *et al.*, 2014b). Among the five heterotrophic bacterial isolates tested (Supporting Information Fig. S7), the stimulation of growth of strain MY3 was remarkable with the addition of mycobacterial isolate B4 (Fig. 3B and Supporting Information Table S4). After growth, formation of aggregates composed of cells of strain MY3 and the mycobacterial isolate B4 was observed (Supporting Information Fig. S8). It remains unclear whether exchanges of metabolites or physical attachment stimulated growth of strain MY3. A key property of *Mycobacterium* cells is the incorporation of highly hydrophobic mycolic acids into cell walls (Marrakchi *et al.*, 2014), which may contribute to the enhanced formation of the aggregates.

Cell surface hydrophobicity is a key factor that influences the rate and degree of attachment of microbial cells to solid surfaces (Donlan, 2002). When strain MY3 was incubated with XAD-7 or vermiculite, after oxidation of 0.5 mM ammonia, 77% and 73% of the cells of strain MY3 were attached to XAD-7 and vermiculite respectively. By contrast, attachment of *Nitrosotenuis chungbukensis* was not significant, and only 18.6% and 20.1% of the cells of *N. europaea* attached to XAD-7 and vermiculite respectively (Fig. 3A). Notably, the growth of strain MY3 increased when attached to the XAD-7 beads (Fig. 3B), whereas the growth rates of the other two isolated terrestrial AOA strains, *Nitrosoarchaeum koreensis* and *N. chungbukensis*, and the soil AOB strain, *N. europaea* ATCC 19718, were not affected (Supporting Information Fig. S9). These observations indicate that physiological properties of cells of strain MY3 in biofilm differ from those of freely suspended cells. Indeed, attached nitrifier cells have been reported to be more active than free-living cells (Bazin *et al.*, 1982; Armstrong and Prosser, 1988; Keen and Prosser, 1988). To further explore the surface properties of strain MY3, the hydrophobicity of the cell surface was directly assayed with the extraction from aqueous media using the aromatic solvent, *p*-xylene (Fig. 3C). After extraction of the cells from cultures, most cells of strain MY3 (ca. 90%) were partitioned into the *p*-xylene phase, whereas most cells of *N. koreensis* (97%), *N. chungbukensis* (94.6%) and *N. europaea* (95.6%) remained in the aqueous phase.

Various genes putatively involved in cell adhesion and the production of EPS were detected in the genome of strain MY3 (Supporting Information Table S6), supporting the strain's potential for biofilm formation and attachment. The presence of multiple families and homologues of glycosyl transferases, glycosyl hydrolases, sialidases and other protein families involved in binding, synthesis and modification of oligo- and polysaccharides and acetamido sugars, as well as their organization in genomic clusters, is indicative of the existence of pathways responsible for the production and modification of EPS and consequently, biofilm formation. Exogenous sugars such as glucose and fructose might be supplementary to EPS production by strain MY3 as implicated in the earlier described carbon substrate tests. Previously it has been reported that biofilm formation and EPS production were enhanced by thermophilic archaeal cells in the presence of saccharides (Rinker and Kelly, 1996; Anton *et al.*, 1988). Supplementation of saccharides caused secretion of exopolysaccharides, which increased propensity to form aggregates of cells. Additionally, a number of predicted membrane proteins with attachment and/or interaction-related domains, such as an expanded family of proteins with YVTN beta-propeller repeats ($n = 9$) (Jing *et al.*, 2002) and lectin-domain proteins ($n = 1$) (Staudt *et al.*, 2004), have been identified in the genome of strain MY3. A comprehensive comparative analysis of the thaumarchaeotal genomic repertoire implicated in EPS production and potential biofilm formation indicates high abundance of gene homologues involved in biofilm formation in strain MY3 (K. Melina, O. Pierre, V. Luis, M. Michael, S. Anja, N. Matthias, W. Wolfram, S. Christa, unpubl. data).

Microorganisms in biofilms are able to degrade hydrophobic pollutants much more readily and extensively than the planktonic counterparts (Dasgupta *et al.*, 2013). Biofilm-liquid interfaces feature enriched concentrations of ammonia and other nutrients due to a large pool of bound ammonia or biomass degradation, relative to the bulk aqueous solution. Thus, the survival and stability of populations of nitrifiers in oil-affected environments are theoretically enhanced by the formation of biofilms. Our finding of the hydrophobicity and a strong potential for surface attachment of strain MY3 may reflect an adaptation to the selective pressure in hydrocarbon-contaminated environments.

Ecological distribution and abundance

The genome of strain MY3 is the largest (3.4 Mb) among cultivated AOA, and the genome contains three

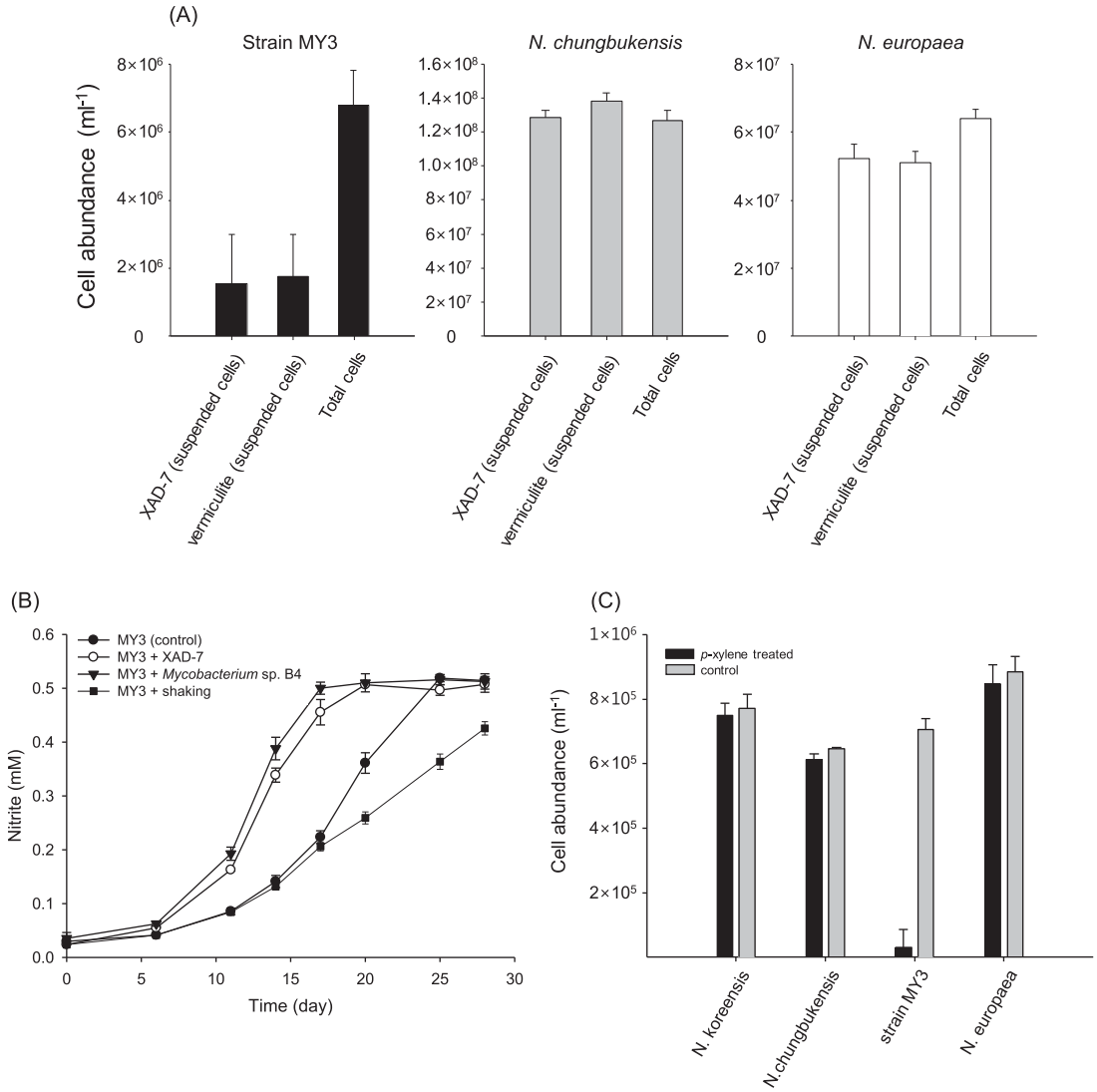

Fig. 3. Assessment of the sorptive and hydrophobic surface properties of strain MY3.
A. Counts of cell concentration of strain MY3, *Ca. N. chungbukensis* and *N. europaea* in the aqueous phase with XAD-7 or vermiculite after oxidation of 0.5 mM ammonia. Cell concentration was measured in the medium after removal of the XAD-7 and vermiculite. For comparison, the respective total cell concentration after growth in the medium without XAD-7 or vermiculite is presented. The initial cell density of strain MY3, *Ca. N. chungbukensis* and *N. europaea* after inoculation was ca. 2.8×10^5, 5.1×10^6, and 3.3×10^6 cells ml^{-1} respectively. The error bars represent the standard deviations based on triplicate experiments.
B. Influence of XAD-7 resin, a mycobacterial isolate (added at a density of ca. 10^5 cells ml^{-1}) and shaking on the growth of strain MY3. For cultivation in shaking condition, the cultures were incubated on a gyratory shaker at 150 rpm in AFM. The initial cell density after inoculation was ca. 2.9×10^5 cells ml^{-1}.
C. Extraction of cells of strain MY3, soil I.1a group AOA (*Ca. N. koreensis* and *Ca. N. chungbukensis*) and *N. europaea* using an aromatic oil, *p*-xylene. Cell concentration remained in the aqueous phase after extraction was measured. DAPI-stained cells were enumerated for total archaeal and bacterial cell counts.

identical copies of the 16S and 23S rRNA genes (Supporting Information Table S2), while the genome only contains single copy of *amoA* and *amoB* gene. This may explain the higher representation of the *Nitrosocosmicus* clade based on the analysis of 16S rRNA gene clones compared with that of *amoA* gene clones has been frequently reported (Mußmann *et al.*, 2011; Xia *et al.*, 2011; Wu and Conrad, 2014). Our results indicate that analysis of 16S rRNA gene-based composition of terrestrial AOA communities must be performed with caution considering the difference in the number of rRNA gene copies per AOA genome. To our knowledge, this is the first report that a member of the *Thaumarchaeota/Aigarcheota/Crenarchaeota/Korearchaeota* (TACK) group (Guy and Ettema, 2011; Spang *et al.*, 2015) carries multiple copies of rRNA genes. rRNA gene-copy number reflects ecological strategies of prokaryotes (Condon *et al.*, 1995): microorganisms with high copy

number of rRNA operon have the ability to quickly respond to environmental changes (Klappenbach *et al.*, 2000).

In conclusion, the properties of strain MY3 suggest a unique hydrophobic niche in terrestrial environments compared with members of the other clades of AOA and AOB. Furthermore, these findings highlight marked differences in basic physiological features in relatively closely related members of the *Nitrosocosmicus* clade, strain MY3 and *N. franklandus*: e.g., growth temperature and sensitivity to ammonia toxicity. Our results suggest that strain MY3 may have an important role in nitrification in biofilms of oil-affected terrestrial environments. It will be interesting to test if the cell surface hydrophobicity is widespread in other AOA of thaumarchaeotal group I.1b. Therefore, based on the phylogenetic and the physiological results of this study, we propose, provisional classification of the new species name for strain MY3 as '*Candidatus* Nitrocosmicus oleophilus' MY3 (see the Supporting Information).

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References

Alves, R.J., Wanek, W., Zappe, A., Richter, A., Svenning, M.M., Schleper, C., and Urich, T. (2013) Nitrification rates in Arctic soils are associated with functionally distinct populations of ammonia-oxidizing archaea. *ISME J* **7**: 1620–1631.

Anton, J., Meseguer, I., and Rodriguez-Valera, F. (1988) Production of an extracellular polysaccharide by *Haloferax mediterranei*. *Appl Environ Microbiol* **54**: 2381–2386.

Armstrong, E.F., and Prosser, J.I. (1988) Growth of *Nitrosomonas europaea* on ammonia-treated vermiculite. *Soil Biol Biochem* **20**: 409–411.

Bazin, M.J., Cox, D.J., and Scott, R.I. (1982) Nitrification in a column reactor: limitations, transient behaviour and effect of growth on a solid substrate. *Soil Biol Biochem* **14**: 477–487.

Condon, C., Liveris, D., Squires, C., Schwartz, I., and Squires, C.L. (1995) rRNA operon multiplicity in *Escherichia coli* and the physiological implications of rrn inactivation. *J Bacteriol* **177**: 4152–4156.

Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., *et al.* (2015) Complete nitrification by *Nitrospira* bacteria. *Nature* **528**: 504–509.

Dasgupta, D., Ghosh, R., and Sengupta, T.K. (2013) Biofilm-mediated enhanced crude oil degradation by newly isolated *pseudomonas* species. *ISRN Biotechnol* 2013: Article ID 250749, 13.

Donlan, R.M. (2002) Biofilms: microbial life on surfaces. *Emerg Infect Dis* **8**: 881–890.

French, E., Kozlowski, J.A., Mukherjee, M., Bullerjahn, G., and Bollmann, A. (2012) Ecophysiological characterization of ammonia-oxidizing archaea and bacteria from freshwater. *Appl Environ Microbiol* **78**: 5773–5780.

Guy, L., and Ettema, T.J. (2011) The archaeal 'TACK' superphylum and the origin of eukaryotes. *Trends Microbiol* **19**: 580–587.

Hatzenpichler, R., Lebedeva, E.V., Spieck, E., Stoecker, K., Richter, A., Daims, H., and Wagner, M. (2008) A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring. *Proc Natl Acad Sci USA* **105**: 2134–2139.

Jeon, C.O., Park, W., Padmanabhan, P., DeRito, C., Snape, J.R., and Madsen, E.L. (2003) Discovery of a bacterium, with distinctive dioxygenase, that is responsible for *in situ* biodegradation in contaminated sediment. *Proc Natl Acad Sci USA* **100**: 13591–13596.

Jing, H., Takagi, J., Liu, J.H., Lindgren, S., Zhang, R.G., Joachimiak, A., *et al.* (2002) Archaeal surface layer proteins contain beta propeller, PKD, and beta helix domains and are related to metazoan cell surface proteins. *Structure* **10**: 1453–1464.

Jung, M.Y., Park, S.J., Min, D., Kim, J.S., Rijpstra, W.I., Sinninghe Damsté, J.S., *et al.* (2011) Enrichment and characterization of an autotrophic ammonia-oxidizing archaeon of mesophilic crenarchaeal group I.1a from an agricultural soil. *Appl Environ Microbiol* **77**: 8635–8647.

Jung, M.Y., Park, S.J., Kim, S.J., Kim, J.G., Sinninghe Damsté, J.S., Jeon, C.O., and Rhee, S.K. (2014a) A mesophilic, autotrophic, ammonia-oxidizing archaeon of thaumarchaeal group I.1a cultivated from a deep oligotrophic soil horizon. *Appl Environ Microbiol* **80**: 3645–3655.

Jung, M.Y., Well, R., Min, D., Giesemann, A., Park, S.J., and Kim, J.G. (2014b) Isotopic signatures of N_2O produced by ammonia-oxidizing archaea from soils. *ISME J* **8**: 1115–1125.

Kasai, Y., Takahata, Y., Hoaki, T., and Watanabe, K. (2005) Physiological and molecular characterization of a microbial community established in unsaturated, petroleum-contaminated soil. *Environ Microbiol* **7**: 806–818.

van Kessel, M.A., Speth, D.R., Albertsen, M., Nielsen, P.H., Op den Camp, H.J., Kartal, B., *et al.* (2015) Complete nitrification by a single microorganism. *Nature* **528**: 555–559.

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

Könneke, M., Schubert, D.M., Brown, P.C., Hügler, M., Standfest, S., Schwander, T., *et al*. (2014) Ammonia-oxidizing archaea use the most energy-efficient aerobic pathway for CO2 fixation. *Proc Natl Acad Sci USA* **111**: 8239–8244.

Keen, G.A., and Prosser, J.I. (1988) The surface growth and activity of *Nitrobacter*. *Microb Ecol* **15**: 21–39.

Kim, B.K., Jung, M.Y., Yu, D.S., Park, S.J., Oh, T.K., Rhee, S.K., and Kim, J.F. (2011) Genome sequence of an ammonia-oxidizing soil archaeon, "*Candidatus* Nitrosoarchaeum koreensis" MY1. *J Bacteriol* **193**: 5539–5540.

Kim, J.G., Jung, M.Y., Park, S.J., Rijpstra, W.I., Sinninghe Damsté, J.S., Madsen, E.L., *et al*. (2012) Cultivation of a highly enriched ammonia-oxidizing archaeon of thaumarchaeotal group I.1b from an agricultural soil. *Environ Microbiol* **14**: 1528–1543.

Kim, J.G., Park, S.J., Sinninghe Damsté, J.S., Schouten, S., Rijpstra, W.I.C., Jung, M.Y., *et al*. (2016) Hydrogen peroxide detoxification is a key mechanism for growth of ammonia-oxidizing archaea. *Proc Natl Acad Sci USA* **113**: 7883–7893.

Klappenbach, J.A., Dunbar, J.M., and Schmidt, T.M. (2000) rRNA operon copy number reflects ecological strategies of bacteria. *Appl Environ Microbiol* **66**: 1328–1333.

Lebedeva, E.V., Hatzenpichler, R., Pelletier, E., Schuster, N., Hauzmayer, S., Bulaev, A., *et al*. (2013) Enrichment and genome sequence of the group I.1a ammonia-oxidizing Archaeon "*Ca*. Nitrosotenuis uzonensis" representing a clade globally distributed in thermal habitats. *PLoS One* **8**: e80835.

Lehtovirta-Morley, L.E., Stoecker, K., Vilcinskas, A., Prosser, J.I., and Nicol, G.W. (2011) Cultivation of an obligate acidophilic ammonia oxidizer from a nitrifying acid soil. *Proc Natl Acad Sci USA* **108**: 15892–15897.

Lehtovirta-Morley, L.E., Ge, C., Ross, J., Yao, H., Nicol, G.W., and Prosser, J.I. (2014) Characterisation of terrestrial acidophilic archaeal ammonia oxidisers and their inhibition and stimulation by organic compounds. *FEMS Microbiol Ecol* **89**: 542–552.

Lehtovirta-Morley, L.E., Ross, J., Hink, L., Weber, E.B., Gubry-Rangin, C., Thion, C., *et al*. (2016) Isolation of '*Candidatus* Nitrosocosmicus franklandus', a novel ureolytic soil archaeal ammonia oxidiser with tolerance to high ammonia concentration. *FEMS Microbiol Ecol* **92**. fiw057.

Madsen, E.L., Sinclair, J.L., and Ghiorse, W.C. (1991) In situ biodegradation: microbiological patterns in a contaminated aquifer. *Science* **252**: 830–833.

Marrakchi, H., Laneelle, M.A., and Daffe, M. (2014) Mycolic acids: structures, biosynthesis, and beyond. *Chem Biol* **21**: 67–85.

Martens-Habbena, W., Berube, P.M., Urakawa, H., de la Torre, J.R., and Stahl, D.A. (2009) Ammonia oxidation kinetics determine niche separation of nitrifying archaea and bacteria. *Nature* **461**: 976–979.

Mosier, A.C., Allen, E.E., Kim, M., Ferriera, S., and Francis, C.A. (2012) Genome sequence of "*Candidatus* Nitrosoarchaeum limnia" BG20, a low-salinity ammonia-oxidizing archaeon from the San Francisco bay estuary. *J Bacteriol* **194**: 2119–2120.

Mußmann, M., Brito, I., Pitcher, A., Sinninghe Damsté, J.S., Hatzenpichler, R., Richter, A., *et al*. (2011) Thaumarchaeotes abundant in refinery nitrifying sludges express *amoA* but are not obligate autotrophic ammonia oxidizers. *Proc Natl Acad Sci USA* **108**: 16771–16776.

Palatinszky, M., Herbold, C., Jehmlich, N., Pogoda, M., Han, P., von Bergen, M., *et al*. (2015) Cyanate as an energy source for nitrifiers. *Nature* **524**: 105–108.

Prosser, J.I., and Nicol, G.W. (2012) Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. *Trends Microbiol* **20**: 523–531.

Qin, W., Amin, S.A., Martens-Habbena, W., Walker, C.B., Urakawa, H., Devol, A.H., *et al*. (2014) Marine ammonia-oxidizing archaeal isolates display obligate mixotrophy and wide ecotypic variation. *Proc Natl Acad Sci USA* **111**: 12504–12509.

Quaiser, A., Ochsenreiter, T., Klenk, H.P., Kletzin, A., Treusch, A.H., Meurer, G., *et al*. (2002) First insight into the genome of an uncultivated crenarchaeote from soil. *Environ Microbiol* **4**: 603–611.

Rinker, K.D., and Kelly, R.M. (1996) Growth physiology of the hyperthermophilic archaeon *Thermococcus litoralis*: development of a sulfur-free defined medium, characterization of an exopolysaccharide, and evidence of biofilm formation. *Appl Environ Microbiol* **62**: 4478–4485.

Sauder, L.A., Peterse, F., Schouten, S., and Neufeld, J.D. (2012) Low-ammonia niche of ammonia-oxidizing archaea in rotating biological contactors of a municipal wastewater treatment plant. *Environ Microbiol* **14**: 2589–2600.

Simon, H.M., Dodsworth, J.A., and Goodman, R.M. (2000) Crenarchaeota colonize terrestrial plant roots. *Environ Microbiol* **2**: 495–505.

Simon, H.M., Jahn, C.E., Bergerud, L.T., Sliwinski, M.K., Weimer, P.J., Willis, D.K., and Goodman, R.M. (2005) Cultivation of mesophilic soil crenarchaeotes in enrichment cultures from plant roots. *Appl Environ Microbiol* **71**: 4751–4760.

Sonthiphand, P., and Limpiyakorn, T. (2011) Change in ammonia-oxidizing microorganisms in enriched nitrifying activated sludge. *Appl Microbiol Biotechnol* **89**: 843–853.

Spang, A., Poehlein, A., Offre, P., Zumbragel, S., Haider, S., Rychlik, N., *et al*. (2012) The genome of the ammonia-oxidizing *Candidatus* Nitrososphaera gargensis: insights into metabolic versatility and environmental adaptations. *Environ Microbiol* **14**: 3122–3145.

Spang, A., Saw, J.H., Jorgensen, S.L., Zaremba-Niedzwiedzka, K., Martijn, J., Lind, A.E., *et al*. (2015) Complex archaea that bridge the gap between prokaryotes and eukaryotes. *Nature* **521**: 173–179.

Staudt, C., Horn, H., Hempel, D.C., and Neu, T.R. (2004) Volumetric measurements of bacterial cells and extracellular polymeric substance glycoconjugates in biofilms. *Biotechnol Bioeng* **88**: 585–592.

Stieglmeier, M., Klingl, A., Alves, R.J., Rittmann, S.K., Melcher, M., Leisch, N., and Schleper, C. (2014) *Nitrososphaera viennensis* gen. nov., sp. nov., an aerobic and mesophilic, ammonia-oxidizing archaeon from soil and a member of the archaeal phylum *Thaumarchaeota*. *Int J Syst Evol Microbiol* **64**: 2738–2752.

de la Torre, J.R., Walker, C.B., Ingalls, A.E., Konneke, M., and Stahl, D.A. (2008) Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol. *Environ Microbiol* **10**: 810–818.

Tourna, M., Stieglmeier, M., Spang, A., Konneke, M., Schintlmeister, A., Urich, T., *et al*. (2011) *Nitrososphaera viennensis*, an ammonia oxidizing archaeon from soil. *Proc Natl Acad Sci USA* **108**: 8420–8425.

Walker, C.B., de la Torre, J.R., Klotz, M.G., Urakawa, H., Pinel, N., Arp, D.J., *et al*. (2010) *Nitrosopumilus maritimus* genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. *Proc Natl Acad Sci USA* **107**: 8818–8823.

Wang, B., Zhao, J., Guo, Z., Ma, J., Xu, H., and Jia, Z. (2015) Differential contributions of ammonia oxidizers and nitrite oxidizers to nitrification in four paddy soils. *ISME J* **9**: 1062–1075.

Weidler, G.W., Gerbl, F.W., and Stan-Lotter, H. (2008) *Crenarchaeota* and their role in the nitrogen cycle in a subsurface radioactive thermal spring in the Austrian Central Alps. *Appl Environ Microbiol* **74**: 5934–5942.

Wu, Y., and Conrad, R. (2014) Ammonia oxidation-dependent growth of group I.1b *Thaumarchaeota* in acidic red soil microcosms. *FEMS Microbiol Ecol* **89**: 127–134.

Xia, W., Zhang, C., Zeng, X., Feng, Y., Weng, J., Lin, X., *et al*. (2011) Autotrophic growth of nitrifying community in an agricultural soil. *ISME J* **5**: 1226–1236.

Xu, M., Schnorr, J., Keibler, B., and Simon, H.M. (2012) Comparative analysis of 16S rRNA and *amoA* genes from archaea selected with organic and inorganic amendments in enrichment culture. *Appl Environ Microbiol* **78**: 2137–2146.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Relationships among ammonia oxidation, nitrite production, and strain MY3 growth. The ammonia and nitrite concentrations were determined colorimetrically. Archaeal cells were counted by FISH. The initial cell density after inoculation was ca. 1.6×10^5 cells ml^{-1}. Initially, ca. 1 mM ammonia was added as an energy source. The error bars represent the standard deviations based on triplicate experiments.

Fig. S2. Characteristics pertinent to the ability of strain MY3 to use urea as a substrate for growth. (A) Correlation between growth of strain MY3 (estimated via nitrite production; open circles), urea loss (solid circles), and cell concentration (solid bars) during growth on medium containing 0.25 mM urea. Error bars represent standard errors. (B) Schematic representation of potential urease gene cluster of strain MY3 compared with those of close relatives of thaumarchaeal group I.1b and *Cenarchaeum symbiosum*.

Fig. S3. Effects of pH (A), temperature (B), ammonia (C) on the ammonia oxidation activity of strain MY3. The ammonia oxidation activity was measured by nitrite accumulation. The initial cell density after inoculation was ca. 3.1×10^5 cells ml^{-1}.

Fig. S4. Comparative phylogenetic analysis of 16S rRNA gene sequences from strain MY3. Branching patterns supported by bootstrap values (1 000 iterations) > 50%, according to the neighbor-joining method, are denoted by those bootstrap values. The strains marked in blue are reference strains or fosmid clones of *Thaumarchaeota*, and the environmental sequences obtained from wastewater treatment plants are in bold. The putative '*Nitrosocosmicus*' clade is red-shaded. The origins of each clone or strain are indicated in parentheses with their accession numbers.

Fig. S5. Effects of addition of different organic compounds on specific growth rate (A), and protein concentration normalized against NO_2^- production (B). Error bars represent the standard error of the mean of triplicate cultures. Control indicates cultures without organic carbon supplemented. The data of *N. viennensis* and strain DDS1 in (A) taken from Touna *et al*. (2011) and Kim *et al*. (2016).

Fig. S6. Comparative phylogenetic analysis of catalase gene sequences from strain MY3. Branching patterns supported by bootstrap values (1 000 iterations) > 50%, according to the neighbor-joining method, are denoted by those bootstrap values. The strains marked in blue are reference strains of *Thaumarchaeota*. The origins of each strain are indicated in parentheses with their protein or genome accession numbers. *, truncated Mn-catalase.

Fig. S7. Effect of co-cultured heterotrophic bacteria on the nitrite production by strain MY3 (A). The effect was also represented by the change in generation time (B). The heterotrophic bacterial strains were isolated from the enrichment culture of strain MY3. Initial cell concentrations of heterotrophic bacteria and strain MY3 were ca. 10^5 cells ml^{-1}. The error bars represent the standard deviations based on triplicate experiments.

Fig. S8. Mixed aggregate of strain MY3 and mycobacterial isolate B4. Merged FISH image with a Cy3-labeled *Archaea*-specific probe (Arc915, blue) and a FAM-labeled *Bacteria*-specific probe (EUB338, green) (scale bar: 2 μm).

Fig. S9. Influence of XAD-7 on the growth of *N. koreensis*, *N. chungbukensis*, and *N. europaea*. The production of nitrite during growth is shown. The error bars represent the standard deviations based on triplicate experiments.

Table S1. Characteristics of the AOA and AOB strains.

Table S2. Putative genes of central carbon metabolism in the genome of strain MY3

Table S3. Putative genes encoding organic transporters in the genome of strain MY3.

Table S4. Bacterial strains isolated from the enrichment culture.

Table S5. Putative genes related to adhesion and involved in production of extracellular polymeric substances.

Table S6. Genome size and number of rRNA gene copies of ammonia- and nitrite-oxidizing microorganisms.

Table S7. Effect of peptides on the growth of strain MY3.

Table S8. Fractional abundance of core GDGTs of strain MY3 growth at 30°C.