Identification and characterization of novel ammonia-oxidizing archaea and bacteria

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dedicated to the scientific idea
Those who do not stop asking silly questions become scientists.

Leon M. Lederman

Study hard what interests you the most in the most undisciplined, irreverent and original manner possible.

All kinds of interesting questions to which science knowledge only adds to the excitement and mystery and the awe of a flower. It only adds. I don’t understand how it subtracts.

Richard P. Feynman

I never did a day’s work in my life. It was all fun.

Thomas A. Edison
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Chapter I

Introduction & Outline
Biogeochemical cycling of nitrogen

**Nitrogen cycling on the early Earth**

Nitrogen is the fifth most abundant element in our solar system and one of the key ingredients of life as we know it. The main sources of nitrogen on the primordial Earth were ammonia (NH$_3$), amino acids, nucleobases and other organic compounds that accumulated on the protoplanet due to planetary accretion and, later, meteoritic infall, volcanic outgassing and abiotic synthesis [1-5]. Although the exact composition of Earth’s primitive atmosphere is still under debate [1, 6-8] there is general agreement that abiotic reactions provided a constant supply of different forms of reduced and oxidized states of nitrogen in the Hadean (>4 Gyr ago) and early Archaean (4-2.5 Gyr ago) environment [see e.g. 1]. Heat-induced geochemical reactions in Earth’s still hot crust and its mantle led to the formation of dinitrogen gas (N$_2$), which was transported into the atmosphere via volcanoes. This was the beginning of a primitive nitrogen cycle, in which thunderstorm- and volcano-induced lightning and annihilating meteorite impacts probably lead to the production of nitric oxide (NO) from atmospheric dinitrogen and carbon dioxide (CO$_2$) [9, 10]. It was suggested that nitric oxide reacted with photochemically produced carboxyl hydride (HCO) to nitroxyld hydride (HNO), which became transported into the ocean by rain to form nitrite (NO$_2^-$) and nitrate (NO$_3^-$) [9, 11]. Although ammonia was also produced by these processes [12], the yield most probably was low and did not significantly contribute to the nitrogen budget [13]. However, in the presence of pyrrhotite (FeS) and hydrogen sulfide (H$_2$S) ammonia forms abiotically from nitrate at temperatures around 100 °C [14]. Such conditions are regularly found in extant deep sea hot vents and probably were also present in primordial hydrothermal systems [1, 14, 15]. Furthermore, it has been shown that at temperatures >25 °C and a pH >7.3 ammonia could have been formed from nitrite via the reduction by ferrous iron (Fe$^{2+}$), which was plentifully available on early Earth [16]. In the atmosphere, photo-oxidation transformed ammonia back to dinitrogen [9] and, by that, closed the cycle [1, 17].

**The evolution of the biologically driven nitrogen-cycle**

Today, the atmosphere contains 78 % dinitrogen gas, representing about half of the total nitrogen present on Earth. While our planet’s mantle and crust are the other main reservoirs for nitrogen, only a minute amount (<0.1 %) is carried within the biosphere [17]. Despite most of the reactions that took place on early Earth are still ongoing, (geological and biological) evolution has transformed our planet from a Hadean nightmare into an oasis of life. Although the exact timing of the biological revolutions that led to the present state is not fully
resolved, the “invention” of anoxygenic photosynthesis most probably paved the way to the nitrogen cycle we know today [8, 17, 18].

The timing of the onset of the main anaerobic processes that are responsible for the reduction of nitrite and nitrate are largely unknown and are heavily disputed [e.g. 18, 19]. As to our current knowledge, nitrite/nitrate can be biologically produced only in the presence of molecular oxygen (O$_2$) that was not present in the Archaean atmosphere [8]. Thus, if anaerobic biological processes using nitrogen-compounds as electron acceptors existed before oxygenic photosynthesis evolved, they probably were only of minor importance [17]. It has been suggested that the rise of anaerobic phototrophs led to the first limitation in available nitrogen in Earth’s history [20] and thus selected for nitrogen-fixation to evolve [21]. However, the early emergence of nitrogen-fixation has been drawn into question, mainly because of its energy-demanding activation step and its underlying gene complexity [9, 19]. In addition, the idea that atmospheric dinitrogen would have suffered a considerable decrease due to the absence of recycling processes at this time is not observed in the geochemical record [9, 19].

As oxygen became available in the surface ocean [22], nitrification, the oxidative part of the nitrogen cycle responsible for the conversion of ammonia to nitrate via nitrite, came into existence. There is evidence from isotopic analyses that as early as 2.5-2.7 Gyr ago nitrification was an active part of biogeochemical cycling [23-25]. Furthermore, it was suggested that at this time either denitrification, the reduction of nitrate to dinitrogen, and/or anaerobic ammonium oxidation (anammox) pathways were already active, suggesting a closed biologically driven nitrogen-cycle in the late Archaean [24]. Consistently, a recent study demonstrated that during only a very brief time-span in the Archaean a massive genetic expansion occurred. Its result was the widespread use of oxygen and redox-sensitive compounds, pointing to a beginning oxygenation of Earth [26]. However, only when biologically available nitrogen was not a limitation any more (~2.3 Gyr ago), dissolved metals (most importantly iron) had been precipitated as metal-oxides (2.4-1.8 Ga ago) and especially once terrestrial plants had evolved (~550 Myr ago) oxygen started to accumulate in the atmosphere [17, 27].

It should be noted that we are still limited in our understanding of the functioning of biogeochemical cycling of elements and many more organisms with yet unknown physiologies are probably hidden in nature. Furthermore, it is unknown whether today’s organisms and metabolisms are (fully) representative for those that led to the biosphere we
live in. Because of these reasons discussions on evolutionary topics must often remain speculative (or “educated guesses”).

**The modern biogeochemical nitrogen-cycle**

Nitrogen can exist in a wide range of different oxidation states (-III in ammonia to +V in nitrate), but only a minor fraction of nitrogen-containing molecules are chemically stable over a long time-span. In contrast, dinitrogen is nearly inert, explaining its accumulation in the atmosphere and its role as original input source of nitrogen for life. In one of the most energy-consuming activation reactions that exist in nature (16 ATP per fixed N\(_2\)), the nitrogenase enzymes of some members of the archaea [28] and bacteria [29] reduce dinitrogen to ammonia. In addition, symbioses of dinitrogen-fixing archaea or bacteria with eukaryotes (e.g. legumes, termites or wood-eating shipworms) also represent a major source of organic nitrogen (Fig. 1) [30, 31]. Despite the existence of a practically endless supply of nitrogen from the atmosphere, most organisms assimilate the more easily and less energy-costly ammonia or nitrate or use organic nitrogen (N\(_{org}\)). Ammonia is released via the mineralization of organic nitrogen by microorganisms (Fig. 1) [32]. It is in equilibrium with ammonium (NH\(_4^+\)) and shifts in this equilibrium occur in dependence of the pH and to a lesser extent the temperature of the environment.

Ammonia and ammonium can be converted aerobically and anaerobically. Under aerobic conditions, ammonia-oxidizing bacteria (AOB) [33-36], the only recently identified ammonia-oxidizing archaea (AOA) [37] as well as at least one member (*Methylococcus capsulatus* strain Bath) of methane-oxidizing bacteria (MOB) [38, 39] gain energy from the oxidation of ammonia to nitrite. While all MOB are able to oxidize ammonia due to the unspecificity of their ammonia/methane-oxidizing enzyme (for a detailed discussion see chapter on the physiology of ammonia oxidizers), *M. capsulatus* is the only known MOB encoding the gene set necessary to live from ammonia as sole source of energy [40-42]. The genome of *M. capsulatus* strain Bath has recently been obtained [40-42], but the organism’s ecological relevance in nitrification is yet unknown.

Besides these autotrophic microbes, some bacteria, fungi and green algae are known to be capable of heterotrophic nitrification, cometabolization-processes that are not coupled to energy-conservation [43]. Some heterotrophic nitrifiers are also capable of aerobic denitrification and, thus, may be relevant for the removal of dissolved nitrogen in some habitats [44, 45]. Most interestingly, the heterotrophic denitrifier *Paracoccus denitrificans*
catalyzes the oxidation of ammonia to nitrite via hydroxylamine as observed for AOB [46]. This is achieved via ammonia monooxygenase and hydroxylamine oxidoreductase enzymes that are, however, not closely related to those of AOB [47, 48]. The importance of heterotrophic, as compared to autotrophic, nitrifiers in the environment is yet not fully resolved, but they are considered to contribute only marginally to nitrogen cycling [33, 49]. This is consistent with the observed low nitrification rates of heterotrophic nitrifiers [50].

![Diagram of nitrogen cycle](image)

**Fig. 1. Schematic representation of the modern, mainly biologically driven nitrogen-cycle on Earth.** Geochemical transformations are not indicated, because for the better part they are of minor importance compared to biologically catalyzed reactions. Abbreviations: Anammox, anaerobic ammonium oxidation; ANRA and DNRA, assimilatory and dissimilatory nitrate/nitrite reduction to ammonium, respectively; AOB, ammonia-oxidizing bacteria; N$_{org}$, organic nitrogen; *, while it is accepted that bacterial ammonia monooxygenase catalyzes the oxidation of ammonia (NH$_3$) to hydroxylamine (NH$_2$OH), the reaction product of its archaeanal counterpart has so far not been identified.

The first step of nitrification is followed by the oxidation of nitrite to nitrate catalyzed by a range of nitrite-oxidizing bacteria (NOB; Fig. 1) [33]. These include members of the α- (genus *Nitrobacter*) [33], β- (*Nitrotoga arctica*) [51], γ- (*Nitrococcus mobilis*) and δ-subclasses (*Nitrospina gracilis*; however, the phylogenetic affiliation of this organism is
disputed) [52, 53] of Proteobacteria, as well as the genus Nitrospira (phylum Nitrospira). As to our current knowledge, microbes affiliated to the genus Nitrospira are the by far most diverse group of NOB in nature. They also constitute the numerically and physiologically most important NOB in most waste water treatment plant sludges [54, 55]. NOB have also been detected in thermophilic sites [56-59] and a moderately thermophilic species has recently been obtained in pure culture [60]. The key enzyme of the metabolism of all yet described NOB is the nitrite oxidoreductase (Nxr) that catalyzes the oxidation of nitrite to nitrate.

Although energetically possible [61], a comammox organism catalyzing the complete oxidation of ammonia to nitrate has not been found so far.

Interestingly, nitrite can also serve as an electron donor for anoxygenic photosynthetic bacteria [62, 63], in a process referred to as phototrophic nitrite-oxidation (Fig. 1). However, the ecological relevance of these bacteria and their contribution to the nitrogen-cycle is yet unclear.

Under oxygen-depleted conditions, ammonium can be anaerobically oxidized to dinitrogen by some members of the Planctomycetes phylum [64-66]. They achieve this by coupling the anaerobic ammonium oxidation (anammox) to the reduction of nitrite to nitric oxide (Fig. 1) [65, 66]. Anammox produces dinitrogen gas via the toxic intermediate hydrazine (N$_2$H$_2$) that is handled in a special intracellular compartment, the anammoxosome [64-67]. The anammox process is of considerable importance in anoxic habitats, such as the Black Sea [68], and can account for 20-70 % of the total dinitrogen production in marine sediments [65, 69, 70].

Nitrite and nitrate produced during nitrification are reduced via nitric oxide and nitrous oxide to dinitrogen by a broad range of canonical (or completely) denitrifying organisms, including archaea, bacteria, fungi, protozoa and foraminifera [71-73]. In a process termed nitrifier denitrification AOB [19, 74] and possibly AOA [75, 76] reductively detoxify nitrite to nitrous oxide via nitric oxide (Fig. 1). In the non-enzymatic process of chemo-denitrification, that only occurs at pH <4.5, nitric acid (HNO$_3$) becomes dismutated, leading to the formation of nitrate and nitric oxide [33].

In highly reducing environments nitrate and nitrite can also be converted via assimilatory or dissimilatory nitrite/nitrate reduction to ammonium (ANRA and DNRA, respectively) [77, 78, 79] (Fig. 1). Furthermore, marine benthic diatoms have been hypothesized to use DNRA to overcome periods of darkness and anoxia [80].
Recently it has been shown that bacteria of the until then enigmatic NC-10 phylum [81-85] and possibly anaerobically methane-oxidizing euryarchaeota (ANME) [85] couple the anaerobic oxidation of methane (AOM) to denitrification (Fig. 1). The NC-10 bacterium “Candidatus Methylomirabilis oxyferans” achieves this via a remarkable mechanism that involves the production of molecular oxygen and thus enables the aerobic oxidation of methane under anaerobic conditions [81].

**Anthropogenic influences on the nitrogen-cycle**

Since the origin of the modern nitrogen-cycle 2.5-2.7 Gyr ago [23, 24] humans are responsible for the probably largest impact on its pathways. Within the last 50 years the world has witnessed a doubling in the world’s population [86-88]. This dramatic increase would not have been possible without the widespread application of the Haber-Bosch process, the industrial counterpart to biological nitrogen fixation [89]. As of 1999, the annual amount of anthropogenically fixed nitrogen equaled ~45 % of the naturally fixed dinitrogen in all terrestrial and marine systems combined [17, 88, 90]. Ammonia from the Haber-Bosch process is used as starting material for the production of a wide variety of goods, including nylon, plastics, resins, glues, food supplements for animals and fish, explosives and, most importantly, nitrogen fertilizers [86-88]. Between the years 1960 and 2000 the use of agricultural fertilizers has increased by 800 % [91] and half of its annual production is used for growing only three crops: wheat, rice and maize [17].

Unfortunately, due to the low nitrogen uptake efficiency of crops ~70 % of the synthetically fixed nitrogen is lost to the atmosphere or washed out from soils before assimilation into biomass can occur [17, 88, 90]. As a result of canonical [73] and nitrifier denitrification [19, 74] nitrous oxide is produced and partly escapes into the atmosphere. There, nitrous oxide has a 300-times higher greenhouse warming potential than carbon dioxide and is thus responsible for 5-7 % of the observed greenhouse effect [92]. In addition, nitrous oxide has detrimental effects on Earth’s ozone (O₃) layer [93]. Methane oxidation rates have also been reported to decrease after nitrogen fertilization, due to the co-metabolization of ammonia by aerobic methane oxidizers [94] and their sensitivity towards nitrite [95]. Together, these two effects can lead to an increased flux of methane into the atmosphere, where it acts as a greenhouse gas. Nitrogen washout from fertilization may further lead to the eutrophication of terrestrial, freshwater and coastal ecosystems, ocean acidification or result in the production of hypoxic or anoxic zones in water bodies [88, 90]. In consequence, the change in pH of marine waters has recently been shown to decrease the nitrification rate of these systems [96]. Besides
severe effects on biodiversity and other environmental consequences, human health is also affected by the introduced nitrogen. Ammonia and nitrite are toxic for aquatic life (and in higher amounts also for humans) and nitrate is suspected to be a factor in cancerogenesis, acquired methemoglobinemia (a disorder characterized by high levels of oxidized hemoglobin) and reproductive diseases [88 and references therein].

To reduce the problems associated with an increased environmental nitrogen load, industrial and municipal waste water treatment plants are used to detoxify and remove anthropogenic nitrogen compounds. In waste water treatment plants spatially or timely separated oxic and anoxic conditions are provided, allowing for nitrification and denitrification and/or anammox to occur, respectively. As result, nitrogen is not only incorporated into the microbial biomass in these reactors but also released as dinitrogen gas.

In addition to the sources mentioned above, fossil fuel burning is an important source of nitric and nitrous oxides [17, 88, 90]. Counter-intuitively, the usage of biofuels does not provide a solution to this problem, as the crops that are currently used for its production are very nitrogen-intensive (~30 % efficiency), and thus the effect of savings in the carbon dioxide budget is counteracted by an increased release of climate-active nitrogen compounds [97].

At the moment, carbon dioxide and nitrous oxide levels are higher than they ever have been within the past 650,000 years, and there is no indication that nitrogen fertilizer use and fossil fuel burning will be significantly reduced until the year 2050 [92]. A huge part of the emissions of these gases stems from human sources (see above) and the consequences for global climate are currently heavily discussed both in science and in the public. Due to the nitrogen cycle’s interconnection with other major nutrient cycles, such as the carbon, sulfur and phosphor cycle [90], any ecological imbalances can (and probably will) have severe effects on Earth’s climate [92, 98].

Microbes will eventually adapt (rather sooner than later) to the changed environmental conditions, but will humans? Besides other actions to be taken, there is an urgent need for more efficient nitrogen fertilizer management, improved land drainage and for a reconsideration of the current usage of biofuels (not least because these crops use potential farmland). Furthermore, the construction of new and more efficient waste water treatment plants, most of all in the developing countries, is essential [92, 98].

A fundamental scientific problem, however, is that quantitative data on many of the nitrogen fluxes described above currently have uncertainties of ±20 %, and some are only known with accuracies of ±50 % [86]. The only way to solve this problem is the intensive study of the
organisms and processes involved. To achieve this, the combined efforts of scientists from many different fields of research are needed.

**Ammonia-oxidizing archaea and bacteria**

**Phylogenetic diversity and environmental distribution**

As nitrite is not observed to accumulate in the environment, the oxidation of ammonia to nitrite is considered to be the rate-limiting step of nitrification. Until the discovery of ammonia-oxidizing archaea (AOA) [37], ammonia-oxidizing bacteria (AOB) were regarded as to be mostly responsible for the aerobic oxidation of ammonia in nature. Originally described by Sergei Winogradsky [35, 36], AOB are considered to be obligate chemolithoautotrophic organisms that use ammonia and carbon dioxide as their sole sources of energy and carbon, respectively [34]. They occur in a wide range of natural habitats and are of crucial importance in human-made systems such as agricultural soils and waste water treatment plants. AOB fall into two phylogenetically distinct groups within the β- and γ-subclasses of *Proteobacteria* (Fig. 2). Currently, five genera of AOB are known: the monophyletic group of β-proteobacterial AOB includes the genera *Nitrosomonas* and *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus* within the lineages *Nitrosomonas* and *Nitrosospira*, respectively. Members of these groups are globally distributed in many environments, including waste water treatment plants. Only a single γ-proteobacterial genus, *Nitrosococcus*, that so far has only been found in marine habitats, has been described [34, 99]. Shortly after the finding of genes encoding subunits of ammonia monooxygenase (Amo), the central enzyme of ammonia oxidizers, associated with archaeal scaffolds obtained from marine surface waters [100] and soil [76, 101], the isolation of the first ammonia-oxidizing archaeon (AOA) was reported [37]. Besides the surprising finding that archaea are involved in the first step of nitrification, *"Candidatus Nitrosopumilus maritimus"* also is the first representative of a globally distributed group of mesophilic archaea obtained in pure culture [37]. Although it had been isolated from a marine water tank of an aquarium, *N. maritimus* proved to be representative for the lineage of marine group I.1a archaea (Fig. 2) [37, 102-104]. Members of this group are ubiquitously distributed in many environments [105-107] and have been demonstrated to represent 20-30% of all microbes in oceans worldwide [108-112]. Most importantly, since its discovery, *amo* and 16S rRNA gene sequences highly similar to those of *N. maritimus* have been reported from a wide variety of marine samples [102-104, 113-116].
Very recently, the group I.1a AOA “*Candidatus Nitrosoarchaeum limnia*” has been enriched from a low-salinity sediment and its genome was nearly completely sequenced via an elegant combination of metagenomics and single-cell sequencing [117]. Furthermore, the marine sponge symbiont “*Candidatus Cenarchaeum symbiosum*” [118] has been shown to encode genes essential for the oxidation of ammonia [119, 120] and is usually regarded as an AOA. It should however be kept in mind that this organism was never demonstrated to actually catalyze the oxidation of ammonia.

Besides these three group I.1a-species, two (moderately) thermophilic AOA, “*Candidatus Nitrososphaera gargensis*” [121; also see chapter II of this thesis] and “*Candidatus Nitrosocaldus yellowstonii*” [122], have been described (Fig. 2). While the former one was the first identified member of group I.1b (“soil group”) archaea, the latter one represents a thermophilic deep-branching lineage (ThAOA, formerly HWCGIII). Very recently, “*Candidatus Nitrososphaera viennensis*”, the first representative of soil-AOA was obtained in axenic culture. Besides these three known lineages of AOA (group I.1a, group I.1b, ThAOA), AmoA-phylogenies suggest that more, yet unidentified AOA groups may be hidden in nature [102, 121, 122]. The recent insights into the phylogeny of AOA will be discussed below in a separate section (see: A third archaeal phylum: *Thaumarchaeota*).

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**Fig. 2.** *16S rRNA/18S rRNA-based phylogenetic tree demonstrating the affiliation of known nitrifiers.* Ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) are labelled in red, while nitrite-oxidizing bacteria (NOB) are highlighted in blue. Asterisks indicate organisms that have been identified and characterized during this thesis. *Candidatus*-status’ of organisms are omitted for improved readability. Note that although *Cenarchaeum symbiosum* has been shown to encode an ammonia monooxygenase in its genome, so far it has not been shown to be an AOA *sensu stricto*. The tree was calculated via a maximum likelihood (ML) algorithm without the use of phylogenetic filters. The scale bar indicates 10 % estimated sequence divergence.
Since their discovery, AOA have been detected by molecular tools in a huge variety of natural and man-made habitats, including many soils [e.g. 123-131], different oceans [e.g. 103, 104, 113-115], estuaries [e.g. 132, 133], fresh and ground waters [e.g. 134-137], marine sediments [e.g. 102, 138], geothermal springs and deep sea hot vents [e.g. 122, 139-141], corals [142], sponges [e.g. 143-145] and waste water treatment plant sludges [e.g. 146-149]. Since the initial reports of thermophilic AOA [121, 122; and chapter II of this thesis), additional studies reported on the presence of AOA in both terrestrial [139, 141, 150-152] and submarine [140, 153] high temperature environments up to 94 °C. In addition, nitrification [139, 154] and in situ expression of archaeal amoA mRNA [150] have been shown to occur at temperatures of 84-85 °C and 44-94 °C, respectively. In contrast, AOB have not yet been demonstrated to grow at elevated temperatures, while the occurrence of thermophilic NOB, mostly Nitrospira, has been reported [56-60]. In waste water treatment plants AOB constitute a significant part of the microbial community and are mostly found as growing in tight colonies constituted of up to many thousands of cells [34, 148, 155-158]. They are commonly found to live in close proximity to NOB [34, 148, 155, 156, 158]. AOB populations in aerobic waste water treatment plant sludges are often dominated by the genus Nitrosomonas, while only few studies report the presence of members of the Nitrosospira lineage [34, 159, 160] Within the last few years also archaeal amoA gene sequences were detected in different waste water treatment plants [146-149, 161, 162]. However, a more detailed analysis that goes beyond the mere detection and enumeration of gene sequences is needed to examine the relative contribution of AOA and AOB to nitrification in these (or other) systems.

In most hitherto studied environments, like in different soil [129, 130, 163] or marine systems [68, 103, 164], AOA were demonstrated to outnumber AOB, which until then were believed to be solely responsible for the aerobic oxidation of ammonia. The relative involvement in nitrification of AOA, as compared to AOB, is still under debate. For soil, the yet best studied habitat regarding this aspect, partly contradicting observations have been reported [124, 125, 128, 130, 131, 165-169], which have nevertheless helped to assemble a preliminary picture on the niche preferences of both groups. Different environmental factors, most importantly low pH, low nutrient concentrations and sulfide-containing habitats, have been postulated to select for AOA over AOB [105]. Most importantly, AOA exhibit a remarkable adaptation to highly oligotrophic conditions. This and other factors will be discussed further below (see next section).
**Physiology of ammonia oxidizers**

The central enzyme in the biochemistry of AOA and AOB is the ammonia monooxygenase (Amo). Amo is a member of the Amo/pMmo/pBmo/pXmo enzyme-group, a diverse family of copper-containing membrane-associated monooxygenases (CuMMOs) that engage in the oxidation of ammonia (Amo) [34, 37, 170-172], methane (pMmo) [173, 174], butane (pBmo) [175] and other, yet unidentified substrates (pXmo) [176]. CuMMOs occur in a wide range of archaea and bacteria, including members of the *Thaumarchaeota* [107, 177, 178], the α-, β- and γ-subclasses of *Proteobacteria* [33, 174, 179], *Verrucomicrobia* [180, 181], *Actinobacteria* [175] and the NC-10 phylum [81]. Bacterial Amo does not exhibit high substrate specificity: methane, carbon monoxide (CO) as well as some aliphatic and aromatic compounds may be oxidized and act as competitive inhibitors of Amo [182, 183]. These substrates can, however, not be used for energy-conservation and do not support the growth of AOB [33, 182]. This low specificity is regarded as reflection of the evolutionary history of Amo, as a member of the Amo/pMmo/pBmo/pXmo enzyme-family [33, 182, 184]. Hence, it has been hypothesized that not the type of CuMMO but the downstream biochemical machinery defines an organism’s substrate specificity [179], and, thus, the mere detection of an amoA-like gene, transcript or protein is insufficient to propose an ammonia-oxidizing function [185]. CuMMOs are heterotrimers and in bacteria are commonly genetically encoded in the canonical subunit order C-A-B. In contrast, in AOA the genes are not clustered or do not occur in this order [117, 120, 186]. Furthermore, it has been shown that the pXmoABC-operon in recently identified γ-proteobacterial methanotrophs also do not follow this “rule” [179].

In ammonia oxidizers the genes *amoA*, *amoB* and *amoC* encode the three subunits of Amo [34, 37, 170]. While in AOB multiple, nearly identical *amoCAB*-copies occur [170], no AOA has been found to encode more than a single copy of *amoA* or *amoB* (two *amoC* copies are regularly found, though) [117, 120, 186]. While AmoA is considered to harbor the active site of the enzyme [187], the function of AmoB is yet not fully resolved. AmoC is regarded to exhibit chaperonic activity on AmoA and AmoB to assist their integration into the cytoplasmic membrane [188]. Furthermore, two additional genes, *amoR* and *amoD* have been identified in *Nitrosococcus oceani*. They were found to be co-transcribed with *amoCAB*, and *amoD* was found to be highly similar to a gene in β-proteobacterial AOB [189]. Their function and exact subcellular location remain elusive. Unfortunately, Amo yet defied all attempts for functional isolation, crystalization and structural analysis.
In AOB the membrane-associated Amo catalyzes the aerobic oxidation of ammonia to hydroxylamine (NH$_2$OH) that is subsequently oxidized to nitrite by the periplasmic hydroxylamine oxidoreductase (Hao) [33] according to the equations:

\[
\begin{align*}
\text{Amo:} & \quad \text{NH}_3 + O_2 + 2 H^+ + 2 e^- \rightarrow \text{NH}_2\text{OH} + H_2O \quad (\Delta G_0' = -120 \text{ kJ mol}^{-1}) \\
\text{Hao:} & \quad \text{NH}_2\text{OH} + H_2O \rightarrow \text{NO}_2^- + 5 H^+ + 4 e^- \quad (\Delta G_0' = -114 \text{ kJ mol}^{-1})
\end{align*}
\]

At standard conditions (pH 7.0, 25 °C) only ~0.7 % of the total ammonia/um is available as ammonia. While it is generally accepted that ammonia and not ammonium is the substrate for bacterial Amo [172], AOA have never been tested in that regard. Furthermore, it has been drawn into question whether archaeal Amo catalyzes the same reaction as its bacterial conterpart, because a Hao-homologue has not been found in any of the sequenced AOA. Thus, either a yet unidentified enzyme substitutes for the lack of Hao in AOA [117, 120, 186] or the archaeal Amo yields a different reaction product. Recently it was suggested that nitroxyl hydride (HNO) may be generated by a monooxygenase reaction of the archaeal Amo. Subsequently, nitroxyl hydride could be oxidized to nitrite via a nitroxyl oxidoreductase (Nxor) [186, 190]. The activation of molecular oxygen for the monooxygenase reaction could also be achieved by nitric oxide, the reaction product of nitrite reductase, which would result in the yield of dinitrogen gas [190; Klotz, unpublished]. Consistent with this view, it was recently reported that archaeal nitrite reductases are expressed under aerobic conditions in soils [75], suggesting a different function of these enzymes in AOA than in their bacterial counterparts. If this model tests true, AOA, in constrast to AOB, would not contribute to the production of the greenhouse gas nitrous oxide. It would also help to explain the occurrence of AOA in suboxic habitats, such as certain marine waters, sediments and thermal springs [68, 105, 114, 190].

In all of the above scenarios two of the four electrons released generate a proton motive force and lead to the conservation of energy via an ATPase, while the other two electrons are required for the Amo-reaction [33, 186]. For both AOA and AOB, the exact amount of synthesized ATP per mol ammonia is unknown, because the amount of electrons available for energy conservation is dependend on the growth stage, the extent of reverse electron flow and other factors, and the electron transport process is yet not fully understood [33, 186]. However, the lack of cytochrome-c proteins and the existence of a high number of genes enconding copper-containing proteins in AOA suggest a different electron transport mechanism [117, 120, 186] as compared to the highly iron-dependent AOB [191, 192].

Bacterial Amo was reported to have an exceptionally high stability \textit{in vivo} [193]. This, together with the constant high ribosomal content of AOB [157, 194], is generally interpreted
as an adaptation of these organisms to the temporally fluctuating substrate concentrations in the environment [33, 34, 195-197]. Until now, data on the stability of Amo and ribosomes in AOA are lacking. AOB are inhibited by a wide range of chemical compounds, including the metal-chelator allylthiourea (C₄H₈N₂S), potassium cyanide (KCN), acetylene (C₂H₂), hydrazine (NH₂NH₂), carbon monoxide, ethanol (CH₃CH₂OH) or acetate (CH₃COOH), but also light [33, 182, 198]. In contrast, only limited data is available for AOA. While allylthiourea was reported to lead to a decrease, but not complete inhibition, of metabolic activity [121 and chapter II of this thesis], acetylene has been demonstrated to cause a total suppression of archaeal ammonia oxidation in soils [128, 199].

Due to nitrifier denitrification AOB [19, 74] and possibly AOA [75, 76] are a major source of nitrous and nitric oxides. Interestingly, addition of nitric oxide and/or gaseous nitrogen dioxide (NO₂) has been shown to result in a strong increase of ammonia-oxidizing activity of Nitrosomonas eutropha, while the removal of nitric oxide resulted in an inhibition of the AOB [33, 200-202]. This may provide an explanation for the sensitivity of ammonia oxidizers to intense aeration or stirring at the beginning of an incubation [33]. Furthermore, nitric oxide and gaseous nitrogen dioxide were reported to have an enhancing effect on ammonia oxidation, but were detrimental to cell yield and energy efficiency [200]. Very interestingly, nitric oxide or nitrogen dioxide may not be regarded as products of nitrifier denitrification, but as an essential agent in the process of ammonia oxidation of AOB [203]. This is based on the observation, that nitrogen dioxide or dinitrogen tetroxide (N₂O₄) can serve as oxidant in the anaerobic ammonia oxidation by N. eutropha [204, 205] that follows the equation:

\[
\text{NH}_3 + \text{N}_2\text{O}_4 + 2 \text{H}^+ + 2 \text{e}^- \rightarrow \text{NH}_2\text{OH} + 2 \text{NO} + \text{H}_2\text{O} \quad (\Delta G_0^{'} = -140 \text{ kJ mol}^{-1})
\]

It is possible that nitrogen dioxide or dinitrogen tetroxide is also involved in the oxidation of ammonia by AOA and/or AOB under aerobic conditions. If nitric oxide is produced by a cell (e.g. via denitrifiers or the reaction shown above), it is rapidly chemically oxidized by molecular oxygen to nitrogen dioxide/dinitrogen tetroxide [33, 206] that is then available for the Amo-reaction. If this is the case for Amo, oxygen does not react directly with ammonia, but this reaction would be mediated by nitrogenous compounds [33, 204, 205]. However, anaerobic oxidation of ammonia was reported to proceed ten-fold slower than the aerobic reaction [204, 205]. So far, no data on the ecological relevance of anaerobic ammonia oxidation mediated by AOB and possibly AOA is available.
One of the most important factors that control the relative distribution of AOA and AOB in the environment [105] is substrate-concentration. Considering the extremely oligotrophic conditions *e.g.* in the open ocean, with nanomolar to sub-nanomolar concentrations of ammonia/um, the high abundance of AOA in these systems [102-104, 113-116] is astonishing. Despite standing pools of compounds are not representative for the ongoing biogeochemical fluxes, these low substrate concentrations have been shown to be the key in understanding the niche differentiation between AOA and AOB [171]. In contrast to several analyzed AOB, *N. maritimus* has been demonstrated to exhibit exceptional high affinities ($K_m = 133$ nM total ammonium) and thresholds ($\leq 10$ nM) to its substrate. Most importantly, these values correlate well with the *in situ* kinetics of the oligotrophic oceans [171] that have been shown to contain high amounts of AOA [102-104, 113-116]. Together, these data suggest that *N. maritimus*-related AOA are responsible for the bulk of ammonia oxidation observed in these habitats, and they call for a re-investigation of the current biogeochemical models that probably underestimate the extent of marine nitrification [171]. The same preference for low ammonia levels has been reported for AOA from different soils [167, 190]. However, the recently obtained soil-isolate *N. viennensis* was found to be adapted to considerably higher concentrations of ammonia (up to 15 mM in contrast to 2-3 mM for *N. maritimus*) [207]. While the AOA’s inhibitory ammonia concentration (20 mM) is comparable to that of the most oligotrophic known AOB (21.4 mM for *N. oligotropha* strain JL21), it is still low compared to the highest ammonia tolerance of AOB (50-1,000 mM) [34, 208-210].

The above observations are in accordance with studies that reported high numbers and activities of AOA in acidic soils [105, 125, 169, 211]. The low pH of these habitats is a driving force in the selection for AOA over AOB as such conditions strongly negatively affect the concentration of available ammonia. To circumvent the problem of low substrate-concentrations many, but not all, species of AOB [212-214] and some AOA [120, 207] encode urease enzymes that allow hydrolyses of urea ($\text{NH}_2\text{CONH}_2$) to carbon dioxide and ammonia. The reaction products can then be used as sources of carbon and energy, respectively, and to regulate the pH in the vicinity of the cell [212-214].

Furthermore, a prevalence of AOA for low ammonia concentrations has also been reported for other habitats [*e.g.* 165, 215, 216]. The adaptation of AOA to oligotrophic conditions is consistent with the idea that most archaea, in contrast to bacteria, are especially adapted to energy-stressed environments [217].
Autotrophy and potential mixotrophy of ammonia oxidizers

Despite both AOA and AOB are generally regarded as lithoautotrophic organisms, important differences in how inorganic carbon gets fixed and organic carbon may be used exist. While AOB rely on the Calvin-Benson-Bassham (Calvin) cycle for carbon fixation [170], a modified version of the 3-hydroxypropionate/4-hydroxybutyrate (3HP/4HB) cycle [218] seems to operate in AOA [117, 186, 207] and other thaumarchaeotes of unknown physiology [120, 219, 220]. In addition, for *N. maritimus* and *C. symbiosum* as well as for marine planktonic archaea a reverse tricarboxylic acid (TCA) cycle for carbon-fixation has been brought into discussion [120, 186, 220]. Recently, archaeal *hcd* and *accA/pccB* gene sequences, both encoding key enzymes of the 3HP/4HB-cycle, have been obtained from different soil samples [123, 131, 167], supporting the idea of a wide-spread distribution of this cycle in AOA. It is, however, important to note that genomic and environmental data must be carefully interpreted, because some enzymes may have different substrate specificities than their homologues in better described species. These specificities often can not be predicted from sequence comparisons alone, but need thorough experimental testing [for a discussion see 221]. Furthermore, heterotrophic growth of *Sulfolobus solfataricus* and *Metallosphaera sedula* that both encode the 3HP/4HB-cycle is well documented [221-223].

The differential preference for carbon fixing-pathways has important consequences for the biochemistry and ecological adaptation of these two groups. Most importantly, in the 3HP/4HB-cycle bicarbonate (also called hydrogen carbonate, HCO$_3^-$) is the fixed carbon-species, while the Calvin-cycle engages in the fixation of carbon dioxide. At neutral and slightly alkaline pH bicarbonate is the preferred carbon-species. In addition, a complete or even rudimentary 3HP/4HB-cycle provides the organism the capability to co-assimilate many different organic compounds, including fermentation products. Ecologically more important may be the ability to use 3-hydroxypropionate (OHCH$_2$CH$_2$COOH), an intermediate in the metabolism of the ubiquitously distributed osmoprotectant dimethylsulfoniopropionate (CH$_3$)$_2$S$^+$CH$_2$CH$_2$COO$^-$) [221, 224]. These points might be of importance for understanding the wide distribution and high abundance of AOA, particularly in marine systems [103, 104, 113-115].

Both carbon fixation pathways are energetically expensive in terms of ATP and NADH/NADPH molecules needed per fixed carbon (7 and 9 ATP, 5 and 6 NADH/NADPH for the synthesis of one pyruvate for the Calvin- and 3HP/4HB-cycle, respectively) [221]. However, the high costs for the synthesis of Calvin-cycle enzymes and their need for many cofactors may not be affordable with the highly oligotrophic lifestyle of AOA [221, 225].
addition, the ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), the carbon-fixing enzyme of the Calvin-cycle, is amongst the most unefficient enzymes known. This for the most part explains why RuBisCO is the most abundant enzyme on Earth [226] and comprises up to 50% of the total protein in some plant and bacterial species [227].

Interestingly, the genomes of AOB [discussed in 170] suggest a potential for heterotrophic or mixotrophic growth. While genes that may enable them to grow on inorganic energy sources other than ammonia are absent in AOB, complete pathways for the oxidation of simple organic compounds (e.g. pyruvate, fructose or glutamate) are present. Transporters for these substrates could however not be identified [170]. For Nitrosomonas europaea, ammonia-dependent growth on fructose has been reported and termed chemolitho-heterotrophy [170, 228]. In addition, some AOB can grow anaerobically using pyruvate as source of energy and nitrite as electron acceptor. Though, compared to aerobic ammonia oxidation, the respective growth rates are much slower [229] and the ecological relevance of this lifestyle is yet unknown.

Although ammonia oxidation is the only known physiology of group I.1a and I.1b archaea so far, mixotrophic and heterotrophic lifestyles have been suggested for members of these lineages [111, 113, 207, 230-234]. A potential for mixotrophic growth has been reported from the analyses of the genomes of N. maritimus and C. symbiosum [119, 120, 186], but these hypotheses are currently not experimentally supported. On the contrary, addition of organic substances has been shown to inhibit ammonia oxidation of N. maritimus [37] and N. yellowstonii [122]. However, in these studies mainly complex substrates (yeast extract and peptone, as well as acetate) that may not be representative for the substrates (and concentrations thereof) present in their respective ecological niches have been tested. Interestingly, these two AOA genomes harbour transporters for different amino acids, di/oligopeptides and glycerol [119, 120, 186], making these compounds ideal candidates for future experiments. This is consistent with studies that demonstrate the ability of natural marine archaeal populations to use amino acids [231] or suggest the use of other organic matter [113, 230, 235]. Cultures of the soil AOA N. viennensis exhibit a 12 times higher cell yield when grown under mixotrophic (pyruvate) instead of purely autotrophic conditions [207]. However, less than 10% of cellular carbon seems to be derived from this heterotrophic substrate under the tested conditions [207]. While key components of the 3HP/4HB-cycle could be identified in the draft genome of N. viennensis, no indications for its dependence on pyruvate as compared to N. maritimus could be found [207].
A membrane lipid specific to ammonia-oxidizing archaea

The main constituents of crenarchaeotal cell membranes are glycerol dibiphytanyl glycerol tetraethers (GDGTs), but other ether-lipids also exist in these organisms. For a long time, the highly stable GDGTs were regarded as an adaptation to the high temperature environments many crenarchaeotes inhabit. Accordingly, (hyper)thermophilic organisms may regulate their membrane integrity by the number of cyclopentane-rings in their GDGTs in dependence on the habitat temperature [236, 237]. Thus, the finding that the same GDGTs are ubiquitously present in mesophilic marine crenarchaeotes (now thaumarchaeotes; see following section) came surprising [238-241]. Furthermore, a GDGT exclusively found in these mesophilic archaea was identified and named crenarchaeol [240, 242]. In addition to its occurrence in the marine system, crenarchaeol has also been demonstrated to be ubiquitously abundant in terrestrial environments [129, 243]. While originally believed to be a specific lipid marker for mesophiles [240, 241], studies later reported the presence of crenarchaeol in many terrestrial hot springs [244-248]. Recently, crenarchaeol has been shown to be an important lipid component of all moderate and thermophilic AOA studied so far [122, 249-251]. Thus, while originally hypothesized to be an crenarchaeotal adaptation to lower temperatures because of its decreased lipid density [242], evidence now points to a wide distribution of this lipid and it seems not be correlated with a certain type of habitat [249].

A third archaeal phylum: Thaumarchaeota

Together with the proposal of Archaea as a separate domain of life, they were divided into two kingdoms/phyla, Crenarchaeota and Euryarchaeota, based on phylogenetic investigations of their 16S rRNA sequences (Fig. 2) [252]. Since then, two additional phyla, the Korarchaeota [253, 254] and Nanoarchaeota [255], were postulated, but their phylogenetic discreteness is still under debate [256, 257]. In the early days of research on this domain, extremophiles dominated the known lifestyles of archaea [258]. However, within the last two decades 16S rRNA gene-surveys indicated the presence of archaea in a wide range of moderate environments, most importantly different terrestrial and marine environments [101, 109, 110, 257, 259-263]. Nevertheless, it took until 2005 before the first mesophilic member of Crenarchaeota, the marine planktonic AOA *N. maritimus*, could be isolated [37]. Most importantly, it became evident that these mesophilic archaea constitute the numerically most abundant archaeal lineage on Earth [101, 102, 108, 109, 111, 112, 230, 259, 264]. Using the genome sequence of *C. symbiosum* [119, 120], Brochier-Armanet *et al*. recently phylogenetically analysed a concatenated dataset of 53 ribosomal proteins that share
homology in archaeal and eukaryal genomes [177]. In contrast to previous phylogenies that focussed on the analysis of 16S rRNA-sequences [101, 257], it was possible to decipher the ancient relationships between the different phylogenetic lineages of Crenarchaeota and Euryarchaeota. Furthermore, the study presented evidence that the “moderate crenarchaeote” C. symbiosum actually is a member of a separate archaeal phylum. The newly discovered lineage branches off before the separation of Crenarchaeota and Euryarchaeota and was classified as Thaumarchaeota (Fig. 2) [177]. Whole genome comparisons and the presence/absence of specific marker genes (e.g. the presence of topoisomerase IB in Thaumarchaeota, in contrast to its absence in all other archaeal genomes) additionally support the notion that Thaumarchaeota form a separate and distinct archaeal lineage [177, 265]. The originality of the genome of C. symbiosum is further supported by its low proportion of archaea-specific clusters of orthologous groups of proteins (COGs) as compared to other archaeal genomes [266]. Unfortunately, at the time the study was performed only one AOA genome was available, which led to an initial reluctance of the scientific community to accept the idea of a novel archaeal phylum. Additional support came from the recent demonstration that C. symbiosum and N. maritimus share a specific set of insertion/deletion sites (indels) in certain tRNA-synthetases and proteins engaged in ribosomal functioning and cell division. Taken together, these signatures clearly distinguish the two microbes from all other known archaea [267].

Until now, ammonia oxidation is the only physiology known for members of the Thaumarchaeota. It is, however, highly probable that their true metabolic diversity has yet escaped our attention. For example, the discovery of giant thaumarchaeotes that may be involved in sulfur-cycling in mangrove swamps demonstrated our lack of knowledge on the biology of members this phylum [268]. Despite its close relatedness to N. maritimus on the 16S rRNA level (>97.7% similarity), Giganthauma karukerense exhibits remarkable differences to this planctonic, sub-micrometer-sized AOA. If these data can be confirmed and extended by future experiments, this newly discovered organism has the potential to shed new light onto this still widely enigmatic phylum [268].

Very recently, the genome sequence of “Candidatus Caldiarchaeum subterraneum” of the HWCGI-group was shown to be cleary distinct from all other archaeal genomes. The study authours suggested that this microbe might belong to a novel lineage (“Aigarchaeota”), but acknowledged that the available data yet do not certify the proposal of a new archaeal phylum [269].
The major goal of this thesis was to extend our knowledge on the diversity and physiology of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB). The main focus was the analyses of moderately thermophilic enrichment cultures that had recently been established by our collaborators Eva Spieck (Hamburg, Germany) and Elena Lebedeva (Moscow, Russia) from Russian geothermal springs. From these cultures I was able to identify and initially characterize two novel species of AOA. The genome analysis of one of these AOA, in addition to two already available genomes, lays further evidence on the recently proposed existence of a third phylum named Thaumarchaeota within the Archaea. I also participated in the analysis of members of this novel lineage in waste water treatment plant systems, in which they were hypothesized to contribute to nitrification. Furthermore, I characterized a novel genus within the betaproteobacteria and analyzed its potential involvement in the oxidation of ammonia in AOA-containing enrichment cultures. For the studies described below, a wide variety of different methods, ranging from classical enrichment cultures and single-cell physiology analyses to metagenomic sequencing and comparative genomics, was used.

Chapter I presented a general introduction to the topics of this thesis. It contains fundamental information on the past and present biogeochemical nitrogen cycle and gives a summary of the current knowledge on AOA and AOB.

Chapter II describes the identification and initial physiological characterization of the AOA “Candidatus Nitrososphaera gargensis”. This microbe represents the first cultivated member of the globally distributed soil group I.1b of archaea and was the first discovered moderately thermophilic ammonia oxidizer. Furthermore, oxidation of ammonia is the only reported physiology for group I.1b archaea so far. It is demonstrated that the metabolic activity of this AOA depends on the concentration of ammonia and that it is highly active at low substrate concentrations, while higher ammonia concentrations, known to have no effect on any known AOB, leads to inhibition of this archaeon. This may point to a niche differentiation between AOA and AOB. Since this first demonstration, the preference of AOA for low ammonia concentrations has been confirmed by other studies.

My contribution to this study: all molecular and microbiological work described, except the initial cultivation and mRNA-analysis, was performed by me. Chemical analyses were performed together with A. Richter. I wrote a draft manuscript that was then revised by M. Wagner and me.

**Chapter III** composes a study that re-investigated the *Thaumarchaeota*-hypothesis. It is based on the examination of the first draft genome sequence of a group I.1b archaeon, *N. gargensis*, and the genomes of *C. symbiosum* and *N. maritimus*, comparative genomics and the phylogenetic analyses of marker proteins. Compelling evidence for the discreteness of this novel archaeal phylum is presented. The study contributes to a deeper understanding of the evolution of the recently acknowledged *Thaumarchaeota* with regard to their phylogeny and basal informational processing. The recent notion that this phylum represents the most abundant archaeal lineage on Earth emphasizes the importance to study both the functional and genetic potential of its members.


My contribution to this study: binning (together with T. Rattei) and initial annotation of metagenomic contigs from the enrichment containing *N. gargensis*, identification and phylogenetic analyses of marker proteins and interpretation of the results. I was also involved in writing the manuscript.

**Chapter IV** reports on the identification and sequencing of the near-complete genome of “*Candidatus Nitrosotenuis uzonensis*”, the first moderately thermophilic group I.1a thaumarchaeote brought into culture. It is demonstrated that this archaeon is a representative
of a lineage of AOA globally distributed in terrestrial high temperature environments. Furthermore, initial insights into the genome of the novel organism are presented.


My contribution to this study: I performed CARD-FISH, phylogenetic and genome analyses, data-interpretation and wrote the presented draft manuscript. I also supervised the process of gathering molecular data that was performed by diploma students during the initial enrichment phase.

In Chapter V a study on the contribution of thaumarchaeotes in nitrification in 52 municipal and industrial waste water treatment plants is described. It is shown that these microbes can only be detected in four of the analyzed plants, in one of which they outnumber AOB by four orders of magnitude and express amoA. By several independent lines of evidence it is demonstrated that the thaumarchaeotes in this plant are not primarily involved in the oxidation of ammonia, but rather make a living from the oxidation of not yet identified organic substances. This study highlights our limited knowledge of thaumarchaeotes and reveals that this phylogenetic group exhibit physiologies other than ammonia oxidation. Furthermore, it contributes to a better understanding of nitrification in waste water treatment plants.


My contribution to this study: archaeal hcd and mcrA gene sequence analyses, N2-fixation assays together with A. Richter, and CARD-FISH analyses together with M. Mußmann. I participated in the discussion of the study results and in figure preparation and commented on the manuscript.
Chapter VI summarizes the data obtained within the past five years on the identification, physiological characterization and preliminary genomic analysis of a novel clade within the beta-subclass of Proteobacteria not closely affiliated with any known ammonia oxidizer. Two species of this novel clade were found to be co-enriched in two AOA-containing enrichment cultures obtained from two geographically distant Russian geothermal springs. Unfortunately, no solid conclusion on the microbes’ physiology can be presented at this point, but hypotheses regarding their metabolism are discussed. The study description will hopefully serve as a useful resource for planning future experiments.


My contribution to this study: all microbiological and molecular-biological experiments described were either performed by me or a diploma student (N. Schuster) under my supervision, except the initial establishment of the ammonia-oxidizing cultures and growth of biomass for metagenome sequencing. I performed preliminary analysis of the obtained genome together with D. Le Paslier and E. Pelletier. A. Galushko provided me with essential input during culturing. NanoSIMS-analyses were performed together with A. Schintlmeister. I also wrote the presented study description.

Chapter VII gives a summary of the presented thesis in English and German.

The Appendix includes Chapter SI that contains an additional publication on multicellular magnetotactic bacteria (MMB). These bacteria were my focus of attention during my participation in the Microbial Diversity course at the Marine Biological Laboratory (Woods Hole, MA) in 2009. In this study, a phototaxis mechanism of MMB from a New England salt marsh is described. It also represents the first report of South-seeking MMB on the Northern hemisphere.

My contribution to this study: experiments and data-analyses were performed in equal contribution with the first author. A draft manuscript was written by the first author and me, which was then edited by all co-authors.

The *Appendix* then lists the **scientific expertise** that I attained during my thesis, including a list of publications, oral presentations and conference abstracts, and ends with my **acknowledgements** and **curriculum vitae**.
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Chapter II

A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring

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A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring

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The recent discovery of ammonia-oxidizing archaea (AOA) dramatically changed our perception of the diversity and evolutionary history of microbes involved in nitrification. In this study, a moderately thermophilic (46°C) ammonia-oxidizing enrichment culture, which had been seeded with biomass from a hot spring, was screened for ammonia oxidizers. Although gene sequences for crenarchaeotal 16S rRNA and two subunits of the ammonia monooxygenase (amoA and amoB) were detected via PCR, no hints for known ammonia-oxidizing bacteria were obtained. Comparative sequence analyses of these gene fragments demonstrated the presence of a single operational taxonomic unit and thus enabled the assignment of the amoA and amoB sequences to the respective 16S rRNA phylotype, which belongs to the widely distributed group I.1b (soil group) of the Crenarchaeota. Catalyzed reporter deposition (CARD)-FISH combined with microautoradiography (MAR) demonstrated metabolic activity of this archaeon in the presence of ammonium. This finding was corroborated by the detection of amoA gene transcripts in the enrichment. CARD-FISH/MAR showed that the moderately thermophilic AOA is highly active at 0.14 and 0.79 mM ammonium and is partially inhibited by a concentration of 3.08 mM. The enriched AOA, which is provisionally classified as “Candidatus Nitrososphaera gargensis,” is the first described thermophilic ammonia oxidizer and the first member of the crenarchaeotal group I.1b for which ammonium oxidation has been verified on a cellular level. Its preference for thermophilic conditions reinvigorates the debate on the thermophilic ancestry of AOA.

ammonia oxidation | archaea | nitrification | thermophile | amoA

Nitrification, the successive microbial oxidation of ammonia via nitrite to nitrate, is a crucial step in the biogeochemical nitrogen cycle, and ammonia-oxidizing microorganisms catalyze the first, rate-limiting step of this process. Until recently, the microbiology of ammonia oxidation was thought to be well understood. Aerobic, chemolithoautotrophic bacteria within the Beta- and Gammaproteobacteria were the only known ammonia-oxidizing microorganisms (1, 2). However, in the last few years, this understanding has been radically changed, first, by the discovery that ammonium can also be oxidized anaerobically by a clade of deep branching planctomycetes (3, 4), and later by the equally surprising cultivation of ammonia-oxidizing archaea (AOA) belonging to the Crenarchaeaota (5). Since then, AOA were found to outnumber ammonia-oxidizing bacteria (AOB) in several terrestrial and marine systems, including different soils (6), the North Sea and Atlantic Ocean (7), the Pacific Ocean (8), and the Black Sea (9). Furthermore, molecular analyses demonstrated that AOA also occur in association with marine sponges (10–13), and amoA sequences related to recognized AOA were retrieved in numerous studies from a wide variety of other habitats (7–9, 14–18), including two moderately thermophilic sites with a temperature below 50°C (19, 20). The latter findings raise the question of whether ammonia oxidation takes place at elevated temperatures and in particular whether AOA thrive under these conditions. This scenario seems theoretically possible because ammonium was detected in concentrations between 2.5 μM and 46.7 mM in thermophilic and hyperthermophilic environments (21, 22) and can be formed thermally (23) or abiotically (24–26). However, this question cannot be answered by mere gene fragment retrieval. Direct evidence for autotrophic ammonia oxidation in these systems via process measurements, in situ demonstration of metabolic activity of thermophilic AOA or AOB, or isolation of these organisms is still lacking. Recent ammonia-oxidizing enrichment cultures were established from microbial mats of the Siberian Garga hot spring, and the temperature optimum for ammonia oxidation of these consortia was estimated to be 50°C (27). Here, one of these ammonia-oxidizing enrichment cultures, which had been maintained for 6 years at 46°C (termed Ga9.2a in the original publication), was screened for ammonia-oxidizing microorganisms with a set of molecular tools. We demonstrate the presence and metabolic activity of AOA in this moderately thermophilic enrichment and provide initial insights into their ecophysiology.

Results

The ammonia-oxidizing enrichment culture was grown aerobically (5.1–5.6 mg of O₂ liter⁻¹; measured in five different replicate flasks at 46°C) and mediated the near stoichiometric conversion of ammonium to nitrite without any detectable nitrate production [supporting information (SI) Fig. 4].

Screening for Known Beta- and Gammaproteobacterial AOB and Nitrite Oxidizers. Initially, the bacterial diversity of the enrichment was monitored by PCR amplification of 16S rRNA gene sequences by using the general bacterial primers 616V and 630R. A total of 42 clones were screened by restriction fragment length polymorphism (RFLP) and assigned to five different patterns; 18 clones representing all RFLP types were sequenced and phylogenetically analyzed (SI Fig. 5). All clones formed a monophyletic group within the Betaproteobacteria, but no 16S rRNA gene sequences related to known AOB or nitrite oxidizers were found. Furthermore, no amplicon was obtained with a 16S rRNA gene-based PCR assay for betaproteobacterial AOB by using primers βamoF and Nso1225R. A combination of these primers

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The authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. EU281317–EU281336).†

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with primers targeting the domain Bacteria (616V or 630R) also did not yield any PCR product. The enrichment was also screened for amoA genes of beta- and gammaproteobacterial AOB, but although the applied assays together target all known AOB, no PCR product was observed. Identical results were observed with the addition of PCR-enhancing substances, suggesting that the negative results were not caused by PCR inhibition. This finding was further confirmed by the addition of suitable control DNA to the enrichment biomass before PCR, which always led to successful amplification (data not shown). Consistent with the PCR results, no FISH signals were detectable after hybridization of the formaldehyde-fixed biomass with a suite of AOB- and nitrite oxidizer-specific probes (SI Table 1).

**Detection of AOA.** Archaeal 16S rRNA genes were PCR-amplified by using two different sets of primers, cloned, RFLP-screened, and sequenced. In total, 46 clones representing all RFLP types were partly or fully sequenced. All 46 sequences were related at >99.1% similarity to each other and assigned to a monophyletic cluster within Crenarchaeota group I.1b (soil group; Fig. 1) that also included partial 16S rRNA gene sequences recently retrieved from a subsurface radioactive thermal spring (20) and a Wisconsin soil (28). In addition, crenarchaeotal amo genes were PCR-amplified from the enrichment, and 19 and 7 clones were sequenced for amoA and amoB, respectively. All cloned amoA and amoB sequences showed similarities of >99.2% and >99.3% to each other, respectively. No amplification product for amoC could be obtained. Consistent with the 16S rRNA tree topology, the AmoA and AmoB sequences retrieved from the enrichment formed a monophyletic branch within the crenarchaeotal I.1b (soil) cluster (Fig. 2A and SI Fig. 6).

To directly link the retrieved crenarchaeotal 16S rRNA gene sequences with single microbial cells in the enrichment, catalyzed reporter deposition (CARD)–FISH with the newly designed clone-specific probe RHGA702 was applied. This probe hybridized exclusively to small cocci of 0.9 μm in diameter, occurring in irregular-shaped microcolonies of 10–50 μm in diameter (Fig. 3). Not all cells of such microcolonies showed a signal after hybridization. Identical results were observed in parallel control experiments with the crenarchaeotal probe Cren512 and the general archaeal probe Arch915. The relative abundance of the crenarchaeotes in the enrichment was determined by an indirect CARD-FISH assay to be 50.4% ± 12.5% (SD) (for details, see SI Results).

**Activity of AOA in the Enrichment.** Transcription of crenarchaeotal amoA was demonstrated with biomass from the enrichment (grown in batch culture with 2.0 mM ammonium in the medium) via RT-PCR by using primers specifically targeting the obtained amoA sequences (amo16F/amo586R) (Fig. 2B). Cloning and sequencing of the RT-PCR product resulted in amoA sequences that were identical to amoA sequences obtained by PCR amplification from the enrichment.

Ammonia-oxidizing activity of the detected crenarchaeote was demonstrated by CARD-FISH/microautoradiography (MAR). The enriched biomass was precultivated at 46°C for 3 h with...
different ammonium concentrations without addition of labeled bicarbonate to allow adaptation of the cells to the incubation conditions. After this step, the ammonium concentration was determined, radioactive bicarbonate was added as sole external carbon source, and the biomass was incubated at 46°C for 12 h. For all ammonium concentrations measured (0.14 mM, 0.79 mM, and 3.08 mM, after preincubation) MAR signals coincided with the AOA microcolonies, but bicarbonate fixation was much lower in the presence of 3 mM ammonium than at lower ammonium concentrations (Fig. 3). For all experiments, chemical analyses showed that 12–22% of the ammonium was removed during the 12-h incubation step, and no cells other than AOA identified by the specific FISH probe showed a positive MAR signal (SI Fig. 7). It should, however, be noted that because the maximum exposure time examined was 15 days, unknown (bacterial) ammonia oxidizers with a very weak activity might have been overlooked. Neither CARD-FISH nor MAR signals were detected for AOA microcolonies in control experiments without added ammonium or with UV-sterilized biomass (data not shown). The absence of CARD-FISH signals without ammonium in the medium suggests that the AOA either reduce their cellular ribosome content in response to starvation and/or that, under these conditions, changes in the cell envelope composition occurred, which negatively affected probe permeability. Addition of 100 μM of the inhibitor allyl-thiourea (AITU) in the presence of 0.76 mM ammonium resulted in strongly decreased MAR signals of the AOA microcolonies (Fig. 3C).

Discussion
This study demonstrates that an ammonia-oxidizing enrichment grown at 46°C contains a crenarchaeote that, according to its 16S rRNA gene, is a member of the group I.1b. This group consists mainly of sequences retrieved from soil and also includes a 16S rRNA gene sequence from a fosmid clone that also carries an ammonia-oxidizing enrichment. CARD-FISH/MAR results after 12-h incubation with radioactive bicarbonate and ammonium concentrations of 3.08 mM (A), 0.79 mM (B), 0.76 mM plus addition of the inhibitor allyl-thiourea (AITU) (C), and 0.14 mM (D) are shown. (Scale bars: 10 μm.) Without addition of ammonium to the medium, neither CARD-FISH nor MAR signals could be observed. Experimental conditions (except for the ammonium concentrations and addition of AITU) and microscopic settings were exactly the same for all experiments shown.
Second, the crenarchaeote in the enrichment was metabolically active at 46°C, while ammonia was oxidized to nitrite as revealed by detection of its amoA mRNA (Fig. 2B). The presence of amoA mRNA is a strong hint that the AOA contributes to the ammonia oxidation in the enrichment by use of its Amo protein. If the detected AOA lived from substrates other than ammonium (or ammonia), e.g., from excretion products of unknown ammonia oxidizers in the enrichment, detection of amoA mRNA would have been very unlikely because transcription of crenarchaeotal and bacterial amoA has been shown to be induced by ammonia (30, 34–36). Finally, incorporation of bicarbonate into single archaeal cells as monitored by CARD-FISH/MAR was observed in the presence of ammonium (Fig. 3) but was absent in medium lacking ammonium.

CARD-FISH/MAR experiments were also applied to gain insights into the ecophysiology of the AOA, although the very limited amount of available living biomass from the enrichment prevented a more encompassing assessment. Interestingly, the AOA was highly active in the presence of 0.14 mM and 0.79 mM ammonium, whereas partial inhibition was observed with 3.08 mM ammonium (Fig. 3). Inhibition of AOB at such a low ammonium concentration has not been reported, to our knowledge, and some AOB strains grow at more than 200 mM ammonium concentration has not been reported, to our knowledge. Apparently, the AOA investigated in this study is even more sensitive to ammonium than members of the Nitrosomonas oligotropha lineage (1, 40), with strain JL-21 as current record holder for ammonium sensitivity whose growth is inhibited at a concentration of 21.4 mM at pH 7.2 (38). The high sensitivity of the AOA to ammonium might be partially explained by the shift of the ammonia/ammonium equilibrium to ammonia at elevated temperatures. However, little is known about the mechanism(s) of inhibition. Recent studies showed that ammonia or ammonium is not only taken up passively by AOB via diffusion, but also that at least some AOB possess active uptake systems (41, 42).

Putative ammonia or ammonium transporters are also encoded that at least some AOB possess active uptake systems (41, 42). Putative ammonia or ammonium transporters are also encoded in the genomes of the AOA “Candidatus Nitrosopumilus maritimus” (http://genome.jgi-psf.org/draft.microbes/nitma/nitma.info.html), relatives of the AOA studied here. Such accumulation mechanisms, if not tightly regulated, might, above a certain extracellular substrate concentration, lead to intracellular ammonia/ammonium concentrations that negatively influence the cytoplasmic pH and thus could result in inhibition. However, little is known about the ammonia oxidation pathways in Archaea (11), other causes for inhibition like the accumulation of toxic intermediates, such as hydroxylamine, cannot be excluded.

Taken together, the CARD-FISH/MAR experiments indicated that the AOA in the enrichment is adapted to moderately low substrate concentrations compatible with the 5.88 µM ammonium measured in the Garga hot spring at the time of sampling (27). Surprisingly, the AOA was not completely inhibited by the addition of 100 µM AIUT (Fig. 3C), a concentration known to completely inhibit ammonia oxidation by AOB (43–45). Potential causes for this residual activity are that (i) during the preincubation, which took place with ammonium but without AIUT, energy storage compounds were built up by the AOA, allowing low rates of bicarbonate incorporation in the absence of a functional Amo; and (ii) crenarchaeotal AmoA is not as susceptible to AIUT as its bacterial counterpart, e.g., by having a higher affinity to or not being as dependent on copper as bacterial AmoA (46).

On the basis of the results of this study, we propose, according to Murray and Schleifer (47) and Murray and Stackebrandt (48), provisional classification of the novel archaeon as “Candidatus Nitrososphaera gargensis.” The short description of “Candidatus Nitrososphaera gargensis” is as follows: phylogenetically related to the crenarchaeotal 16S rRNA sequence cluster II.b (soil group), not isolated; enriched from the Garga hot spring in the Burят Republic (Russia); cocci have a diameter of 0.9 ± 0.3 µm (but note that cell morphology might be slightly altered because of the CARD-FISH procedure) in irregular shaped microcolonies; basis of assignment: amoA, amoB, and 16S rRNA gene sequences (GenBank accession nos. EU281317, EU281322, and EU281334) and detection by the phyotype-specific oligonucleotide probe RHGA702 ((5'-GTG GTCT TC GGTT GGA TCA-3') complementary to helix 24a of the 16S rRNA, aerobic chemolithoautotrophic moderately thermophilic ammonia oxidizer [on the basis of the proposal by Reysenbach and Shock (22) that thermophily starts at 45°C], partially inhibited by ammonium in a concentration of 3.08 mM at pH 7.4 and 46°C.

The discovery of the moderately thermophilic “Candidatus Nitrososphaera gargensis” within the group 1.b is noteworthy as it represents the first organism within this group for which, on a cellular level, ammonia oxidation has been shown. Furthermore, all previously described AOA and AOB are mesophiles. It should be mentioned that enrichment of a thermophilic ammonia oxidizer capable of growth at 55°C was reported from geothermal springs of Kamchatka (Russia), but the obtained cultures were unstable and the putative ammonia oxidizers were not identified beyond a morphological description (49). The discovery of a thermophilic AOA adds to our picture of biogeochemical nitrogen cycling in thermophilic environments for which nitrite oxidation, nitrate ammonification, denitrification, and nitrogen fixation have already been reported (23, 50, 51). Furthermore, a thermophilic origin was hypothesized for anaerobic ammonium oxidation as well (52). The existence of thermophilic AOA is also consistent with the previously postulated idea that the mesophilic crenarchaeotes are descendants of ancestral thermophiles (53) and thus suggests that archaeal ammonia oxidation evolved under thermophilic conditions with the mesophilic lifestyles exemplified by soil or marine AOA likely representing independent, secondary adaptations to lower temperatures. Ammonia oxidation as an ancient form of energy conservation is consistent with the postulated early earth chemically driven nitrogen cycle (54), which provides for the formation of ammonia at high temperatures (24–26).

Materials and Methods

Chemical Analyses of the Enrichment. The concentration of ammonium was measured by fluorescence detection according to Corbin (55) after precolumn derivatization with OPA reagent and HPLC separation. Nitrite and nitrate concentrations were determined quantitatively by UV detection after ion-pair chromatography on a Hypersil ODS C18 column (56). The oxygen concentration in enrichment flasks was determined with an electrode (OXI 96; Nova Analytics).

Amplification, Cloning, and Phylogenetic Analyses of 16S rRNA and amo Genes. PCR amplifications of archaeal and bacterial 16S rRNA and amo genes were performed directly from harvested cells (diluted in 0.9% NaCl) from an ammonia-oxidizing enrichment from the Garga hot spring in the Burjurat Republic, Russia (27), by using primers and annealing temperatures as listed in Table 2. The time of analyses, this enrichment had been maintained for 6 years but had not been screened for ammonia oxidizers with nucleic acid-based methods. All PCRs started with an initial heating step at 95°C for 5–7 min to induce cell lysis. PCR-enhancing substances (betaine, bovine serum albumin, dimethyl sulfoxide, or formamide) were added according to the literature (57, 58). Cloning and sequencing were done as described in ref. 59. The diversity of sequences within the clone libraries was screened with RFLP, by using the enzyme MspI (Fermentas Life Sciences Inc.). From each resulting RFLP pattern, at least one representative clone was sequenced. Sequencing was performed by using the BigDye Terminator Cycle Sequencing Kit v3.1 and the ABI 3130xl Sequencer (Applied Biosystems) following the manufacturer’s protocols. Obtained sequences were phylogenetically analyzed by using the software programs ARB (60) and PHYLIP (61) with comprehensive databases that contained 16S rRNA and all available archaeal amo sequences, respectively.
RT-PCR of Crenarchaeotal amoA mRNA. Cells were harvested from the enrichment by centrifugation and were stored in RNAlater (Ambion) at 4°C until further processing. Total RNA was isolated by using TRizol (Invitrogen) according to the manufacturer’s instructions with the following modifications: RNA was precipitated overnight at −20°C in the presence of 5 μg of glycogen (Sigma). After incubation with DNsase (Sigma), reverse transcription was carried out by using the Revert Aid First Strand cDNA Kit (Fermentas Inc.) according to the manufacturer’s instructions with the clone-specific primer amoS86R (5’-AGC CAC GGA AAG TGA CAG-3’), which had been designed by using ARB. cDNA was amplified by using clone-specific primers amo16F (5’-H11032 GAATTC ATG GAG TCT TCC TCG A-3’). Amplification was performed according to the manufacturer’s instructions with the clone-specific primer amo16F (5’-H11032 GAATTC ATG GAG TCT TCC TCG A-3’), which had been designed by using ARB. cDNA was amplified by using clone-specific primers amo16F (5’-H11032 GAATTC ATG GAG TCT TCC TCG A-3’) and amoS86R (length of amplification: 570 bp) with thermal cycling as follows: initial denaturation at 94°C for 4 min followed by 40 cycles of denaturation at 94°C for 40 s, annealing at 60°C for 30 s, and elongation at 72°C for 40 s, followed by a final elongation step at 72°C for 10 min. Specificity of amplification was confirmed by cloning and sequencing of the amplification product. Absence of contaminating DNA in the RNA extract was demonstrated by performing the aforesaid PCR without the initial reverse transcription step.

FISH and CARD-FISH. To visualize AOB and nitrite oxidizers in the enrichment, FISH was performed as described by Daims et al. (62) by using probes listed in SI Table 1. No unspecific labeling of cells was observed in any sample by using the nonsense probe. After FISH, cells were stained at 4°C with the DNA-binding dye at 1 μg ml⁻¹. Microscopic observation and documentation were accomplished by using a LSM 510 scanning confocal microscope (Zeiss) and the included software.

To visualize AOA in situ, a specific 16S rRNA-targeting FISH-probe was designed by using ARB. Probe RHGA702 (SI Table 1) targeted all crenarchaeotal 16S rRNA sequences obtained from the enrichment but has at least one mismatch to other sequences in the ARB database including all published sequences from Crenarchaeota. For more details regarding this probe, please refer to probeBase (63) at http://www.microbial-ecology.net/probebase. HPLC-purified and lyophilized HRP-conjugated oligonucleotides were purchased from Thermo Electron. For CARD-FISH analyses, ethanol-fixed biomass was immobilized on slides and dehydrated by an increasing ethanol series (50%, 80%, and 96% for 3 min each). Afterward, the slide was dipped into 0.2% agarose (in distilled water) and left to dry at 30°C. Then, permeabilization of cell walls was performed by using 10-min incubation with proteinase K (15 μg ml⁻¹; Sigma). This and all other incubation and washing steps were done at room temperature if not stated otherwise. After permeabilization, the slides were washed for 1 min in distilled water. Subsequently, slides were incubated in 0.1 M hydrochloric acid for 20 min to destroy remaining proteinase K. To inactivate endogenous peroxidases, cells were incubated in a solution of 0.15% hydrogen peroxide in methanol for 30 min. Finally, the slides were washed two times in distilled water for 1 min. The biomass was covered with 20 μl of hybridization buffer (64) at a probe concentration of 0.16 ng μl⁻¹ (65). Hybridization was performed for 46°C for 2.5 h by using the probes Arch19S, Cren152, and RHGA702 (10% formamide) in separate experiments (SI Table 1). By using a “nonsense” probe (SI Table 1), no unspecific labeling of cells could be observed. After hybridization, slides were washed in prewarmed washing buffer (64) at 48°C for 15 min. Before tyramide signal amplification, the slides were dipped into distilled water (4°C) and washed for 15 min in 1× PBS. Excess liquid was removed by tapping the slides, and the slides were incubated at 46°C for 45 min with substrate mix by using 1:1,000 parts fluorescein-labeled tyramide (65). Subsequently, the slides were washed in 1× PBS for 10 min and distilled water for 1 min, respectively. Finally, the slides were DAPI-stained and evaluated microscopically as described above. The diameter of the AOA was determined quantitatively by using a stock solution of 10 μM NaH4Cl in a total volume of 3 ml of growth medium. Two biological replicates were performed for each of these experiments. Furthermore, control experiments were performed without addition of ammonium as well as with UV-sterilized biomass to check for physiological activity without added substrate and for chemophoresis, respectively. For all experiments, the biomass was incubated in vertically fixed vials under aerobic conditions for 3 h at 46°C and was shaken at 110 rpm in a water bath. After this preincubation step, which was included to allow the cells to re-adapt to elevated temperature conditions, a subsample was taken (to determine the ammonium concentration via ion exchange chromatography with chemically suppressed conductivity detection) and 10 μCl⁻¹ [3H]bicarbonate (Banke Laboratory Products) was added to each vial. Subsequently, the biomass was incubated for 12 h under the conditions described above. In addition, 100 μM of the inhibitor AITU (fluka) was added after the preincubation to one of the vials containing 0.76 mm ammonium after preincubation. After incubation, biomass was fixed with ETOH or form-aldehyde as described by Daims et al. (62). CARD-FISH and subsequent DAPI staining were performed as described above. The hybridized samples were dipped in preheated (48°C) LM-1 emulsion (Amersham), exposed for 12–15 days at 4°C in the dark and developed in Kodak D19 (40 g liter⁻¹ of distilled water) before microscopic examination. For each condition and biological replicate, three microautoradiography slides were analyzed and at least 10 AOA microcolonies were examined per slide.

ACKNOWLEDGMENTS. We thank Dave Stahl, Christa Schleper, Alex Loy, and Ingo Schmidt for helpful discussions, and Marc Müßmann for advice with CARD-FISH. Eberhard Bock is acknowledged for financial support of the Baikal expedition, and we thank Peter-Georg Joza for help with the chemical analyses. Roland Hatenzelich is recipient of a DOC (Ph.D.) fellowship of the Austrian Academy of Sciences at the Department of Microbiology, University of Vienna, Austria. This work was partly supported by the Deutsche Forschungsgemeinschaft (Grant SP 667/1–1 to E.V.L.).
Supporting information

Fig. 4. Ammonium oxidation and nitrite production in the enrichment culture over a period of 22 days. The enrichment was inoculated with 10 vol % of the parent enrichment culture causing the initial nitrite concentration of 266 µM. No nitrate production was detectable during the experiment confirming the absence of active nitrite oxidizing bacteria. Interestingly, the ammonium concentration declined in the beginning of the experiment without a concomitant nitrite production indicating either accumulation of ammonium inside ammonia-oxidizing cells [as recently described by Schmidt et al. [Schmidt I, Look C, Bock E, Jetten MS (2004) Microbiology 150:1405-1412]] or chemical loss of ammonium.

Fig. 5. 16S rRNA-based phylogenetic maximum likelihood tree showing the affiliation of the 18 bacterial 16S rRNA gene fragments retrieved from the enrichment. Recognized betaproteobacterial AOB and nitrite oxidizers are in bold. Scale bar represents 5% estimated sequence divergence. Dashed lines denote sequences less than 1,300 nt in length, which were added to the tree by the parsimony interactive tool of ARB without changing the overall tree topology.

Fig. 6. Phylogenetic analyses of AmoA and AmoB of "Candidatus Nitrososphaera gargensis." (A) AmoA evolutionary distance (Fitch) tree showing the phylogenetic positioning of the thermophilic AOA studied (in bold) within the I.1b (soil) cluster. For phylogenetic analyses 188 aa positions were considered. Sequences obtained from thermophilic environments are labeled in red, while those from mesophilic habitats are shown in blue. White and black dots indicate bootstrap (100 iterations) support of >70% and >90%, respectively. Dashed lines indicate short sequences that were added after construction of the tree. Asterisks mark organisms for which the 16S rRNA gene is known. Numbers in parentheses give the number of sequences within a group. Scale bar represents 5% estimated sequence divergence. (B) AmoB evolutionary distance (Fitch) tree constructed considering 143 aa positions. Labels are as for A. Scale bar represents 10% estimated sequence divergence.
Fig. 7. Combination of SYBRgreen staining, CARD-FISH and MAR, demonstrating that only actively metabolizing AOA in the thermophilic enrichment appear MAR-positive. AOA were identified by CARD-FISH, and subsequently, CARD-FISH-negative cells in the vicinity of the AOA colonies were stained by addition of Citifluor containing the nucleic acid binding dye SYBRgreen. For all replicate slides and all experiments, no MAR-positive cells other than AOA could be detected. (A) MAR signal above AOA labeled by CARD-FISH at a concentration of 0.79 mM ammonium. (B and C) Non-AOA cells stained by SYBRgreen from the same experiment. Silver grains are evenly distributed over the entire microscopic field and no accumulation of silver grains can be observed on top of these cells. All bars represent 10 µm and all MAR images were recorded with the same microscope settings. The relatively high background MAR signal is caused by the long exposure time of 12 days.

Table 1. Oligonucleotide probes used in FISH experiments

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence (5’-3’)</th>
<th>Specificity</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338</td>
<td>GCT GCC TCC CGT AGG AGT</td>
<td>most Bacteria</td>
<td>(1)</td>
</tr>
<tr>
<td>EUB338 II</td>
<td>GCA GCC ACC CGT AGG TGT</td>
<td>Planctomycetales</td>
<td>(2)</td>
</tr>
<tr>
<td>EUB338 III</td>
<td>GCT GCC ACC CGT AGG TGT</td>
<td>Verrucomirobiales</td>
<td>(2)</td>
</tr>
<tr>
<td>Arch915</td>
<td>GTG CTC CCC CGC CAA TTC CT</td>
<td>domain Archaea</td>
<td>(3)</td>
</tr>
<tr>
<td>Cren512</td>
<td>CGG CGG CTG ACA CCA G</td>
<td>most Crenarchaeota</td>
<td>(4)</td>
</tr>
<tr>
<td>Bet42a</td>
<td>GCC TTC CCA CTT CGT TT</td>
<td>Betaproteobacteria</td>
<td>(5)</td>
</tr>
<tr>
<td>Gam42a</td>
<td>GCC TTC CCA CAT CGT TT</td>
<td>Gammaproteobacteria</td>
<td>(5)</td>
</tr>
<tr>
<td>Pla46</td>
<td>GAC TTG CAT GCC TAA TCC</td>
<td>Planctomycetales</td>
<td>(6)</td>
</tr>
<tr>
<td>Nso1225</td>
<td>CGC CAT TGT ATT ACG TGT GA</td>
<td>betaproteobacterial ammonia oxidizing bacteria</td>
<td>(7)</td>
</tr>
<tr>
<td>Nso190</td>
<td>CGA TCC CCT GCT TTT CTC C</td>
<td>betaproteobacterial ammonia oxidizing bacteria</td>
<td>(7)</td>
</tr>
<tr>
<td>Nsm156</td>
<td>TAT TAG CAC ATC TTT CGA T</td>
<td>Nitrosomonas spp., Nitrosococcus mobilis</td>
<td>(7)</td>
</tr>
<tr>
<td>Nsv443</td>
<td>CCG TGA CCG TTT CGT TCC G</td>
<td>Nitrosospira spp.</td>
<td>(7)</td>
</tr>
<tr>
<td>NEU</td>
<td>CCC CTC TGC TGC ACT CTA</td>
<td>most halophilic and halotolerant Nitrosomonas spp.</td>
<td>(8)</td>
</tr>
<tr>
<td>CTE</td>
<td>TTC CAT CCC CCT CTG CCG</td>
<td>Comamonas spp., Acidovorax spp., Hydrogenophaga spp., Aquaspirillum spp.</td>
<td>(9)</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>-----</td>
</tr>
<tr>
<td>cluster6a192</td>
<td>CTT TCG ATC CCC TAC TTT CC</td>
<td>N. oligotropha lineage (cluster 6a)</td>
<td>(10)</td>
</tr>
<tr>
<td>cluster6a192comp</td>
<td>CTT TCG ATC CCC TGC TTC C</td>
<td>N. oligotropha lineage (cluster 6a)</td>
<td>(10)</td>
</tr>
<tr>
<td>Ncmob</td>
<td>TCC TCA GAG ACT ACG CGG</td>
<td>Nitrosococcus mobilis</td>
<td>(11)</td>
</tr>
<tr>
<td>Ntspa662</td>
<td>GGA ATT CCG CGC TCC TCT</td>
<td>genus Nitrospira</td>
<td>(12)</td>
</tr>
<tr>
<td>Ntspa662comp</td>
<td>GGA ATT CCG CTC TCC TCT</td>
<td>competitor for Ntspa662</td>
<td>(12)</td>
</tr>
<tr>
<td>NIT3</td>
<td>CCT GTG CTC CAT GCT CCG</td>
<td>Nitrobacter spp.</td>
<td>(13)</td>
</tr>
<tr>
<td>NIT3comp</td>
<td>CCT GTG CTC CAG GCT CCG</td>
<td>competitor for NIT3</td>
<td>(13)</td>
</tr>
<tr>
<td>RHGA702</td>
<td>GTG GTC TCC GGT GGA TCA</td>
<td>&quot;Candidatus Nitrososphaera gargensis&quot;</td>
<td></td>
</tr>
<tr>
<td>Nonsense</td>
<td>AGA GAG AGA GAG AGA GAG</td>
<td>(test for unspecific binding)</td>
<td></td>
</tr>
<tr>
<td>NonEUB</td>
<td>ACT CCT ACG GGA GGC AGC</td>
<td>(test for unspecific binding; complementary to EUB338)</td>
<td>(14)</td>
</tr>
</tbody>
</table>


**Table 2. Primers used for PCR-amplifications of archaeal and bacterial 16S rRNA and *amo* genes**

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Annealing temperature</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Archaea</td>
<td>Arch21F</td>
<td>56</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Arch958R</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1492R</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Bacteria</td>
<td>616V</td>
<td>54</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>630R</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Betaproteobacterial AOB</td>
<td>βamoF</td>
<td>60</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Nso1225R</td>
<td></td>
<td>5, 6</td>
</tr>
<tr>
<td>Crenarchaeotal <em>amoA</em></td>
<td>CrenAmo1F</td>
<td>54</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>CrenAmo1R</td>
<td></td>
<td>7</td>
</tr>
</tbody>
</table>
In all PCR-amplifications a cycle number of 35 was used.


SI Results

Quantification of AOA in the Enrichment via CARD-FISH. The relative abundance of AOA in the enrichment was determined indirectly, because direct counting after AOA-specific CARD-FISH was impossible, as the CARD-FISH protocol for AOA involves a proteinase K treatment causing lysis of some bacteria in the enrichment (note that the AOA in the enrichment could not be reliably detected with monolabeled fluorescent oligonucleotide probes; data not shown). Therefore, we determined the number of bacterial cells by CARD-FISH with the EUB338 probe set using a modified protocol with a lysozyme (5 mg ml\(^{-1}\) in TE buffer; incubation for 60 min at 37°C) instead of a proteinase treatment and determined the number of DAPI stained cells which were not detectable by this assay. This number was considered as an reliable estimate of the relative AOA abundance in the enrichment, as (i) archaea are lysozyme resistant (and thus not affected by the assay), (ii) only one archaeal OTU is present in the enrichment, (iii) the DAPI-positive, bacterial CARD-FISH-negative cells had the same coccoid morphology and size as the AOA, and (iv) eukaryotes were neither detectable by a specific FISH assay with probe EUK516 (1) nor by electron microscopy. The actual AOA number might be slightly lower, because inactive bacteria with a very low ribosome content will not be detected by CARD-FISH. In total, more than 1,100 DAPI-stained cells were analyzed and 21 microscopic fields were counted.

Figure S1.
Figure S2.
Figure S3.
Figure S4.
Chapter III

Distinct gene set in two different lineages of ammonia-oxidizing archaea supports the phylum *Thaumarchaeota*

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Distinct gene set in two different lineages of ammonia-oxidizing archaea supports the phylum Thaumarchaeota

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Globally distributed archaea comprising ammonia oxidizers of moderate terrestrial and marine environments are considered the most abundant archaeal organisms on Earth. Based on 16S rRNA phylogeny, initial assignment of these archaea was to the Crenarchaeota. By contrast, features of the first genome sequence from a member of this group suggested that they belong to a novel phylum, the Thaumarchaeota. Here, we re-investigate the Thaumarchaeota hypothesis by including two newly available genomes, that of the marine ammonia oxidizer \emph{Nitrosopumilus maritimus} and that of \emph{Nitroso-

\textit{sphaera gargensis}, a representative of another evolutionary lineage within this group predominantly detected in terrestrial environments. Phylogenetic studies based on r-proteins and other core genes, as well as comparative genomics, confirm the assignment of these organisms to a separate phylum and reveal a Thaumarchaeota-specific set of core informational processing genes, as well as potentially ancestral features of the archaea.

How many archaeal phyla exist?

Since their recognition as a separate domain of life \cite{1,2} from Eukarya and Bacteria, the Archaea have played an important part in models of the early evolution of cellular life forms \cite{3–5}. In particular, the information-processing machineries of Archaea are considered ancestral forms of the more complex replication, transcription and translation machineries of the eukaryotic cell \cite{6}. To be able to infer early traits (features of the last archaeal common ancestor, LACA) and to study the evolution of metabolism and information processing, it is essential to recognize the diversity within the domain and in particular deeply branching lineages.

Carl Woese and collaborators pointed out that 16S rRNA sequences supported a deep split within the Archaea forming two major lineages (kingdoms, \textit{sensu} Woese \cite{2}), the Crenarchaeota and Euryarchaeota. Crenarchaeota (from Greek for spring or fount \cite{2}) were a potential ancestral branch exclusively represented by hyperthermophilic organisms and the term Euryarchaeota (from Greek for broad, wide) insinuated the global distribution and physiological diversity of its halophilic, methanogenic and thermophilic members. The bipartite separation was supported by several characteristic genetic features, (e.g. the cell division protein FtsZ and histones in Euryarchaeota and specific ribosomal proteins in Crenarchaeota). Interestingly, since 1990 all but two of the newly described species of archaea (~330 in total, \url{www.dsmz.de}) belong to one of the two archaeal kingdoms or phyla (\textit{sensu} Bergey's manual \cite{7}) based on their 16S rRNA phylogeny. In the past decade, two additional archaeal phyla, the Korarchaeota \cite{8,9} and Nanoarchaeota \cite{10}, were postulated based on the 16S rRNA phylogeny of enrichments of Candidatus \emph{Korarchaeum cryptophillum} and of the obligate symbiont Candidatus \emph{Nanoarchaeum equitans}, respectively. However, analyses of different phylogenetic marker molecules were inconsistent and thus the phylogenetic placement of these organisms (in particular that of \emph{Nanoarchaeum}) is still under debate \cite{9,11} (Figure 1a,b).

With the discovery of ammonia-oxidizing archaea (AOA), accumulating evidence indicates that the archael tree indeed comprises more than two major lineages \cite{12}. The discovery of these organisms was initially based on environmentally retrieved 16S rRNA sequences, which placed them as a sister group of the Crenarchaeota (Figure 1b) \cite{13,14}, suggesting that these archaea might have ancestors in hot environments and only later radiated into moderate environments. The AOA have since been referred to as Crenarchaeota in all subsequent 16S.
rRNA-based diversity studies. Using the first available genome sequence of this group, that of Cenarchaeum symbiosum, a marine sponge symbiont [15,16], Brochier-Armanet et al. recently analysed a concatenated data set of 53 ribosomal proteins shared by Archaea and Eukarya [12]. The resulting tree, in contrast to rRNA phylogeny [17,18], not only resolved the ancient division between Crenarchaeota and Euryarchaeota, but also suggested that the ‘moderate Crenarchaeota’ including the AOA actually constitute a separate phylum of the Archaea that branched off before the separation of Crenarchaeota and Euryarchaeota.

The name Thaumarchaeota (from the Greek word thaumas for wonder) was proposed for the new phylum [12]. However, the phylum-level status of the Thaumarchaeota was based on the analysis of a single genome of a marine archean (and a few environmental sequences) and thus the inclusion of additional AOA representing different phylogenetic lineages is important.

**Novel ammonia oxidizers support the ribosomal protein trees**

Gene signatures of Thaumarchaeota, including 16S rRNA genes and genes encoding key enzymes of ammonia oxidation, have been detected in moderate environments, such as soils [19–21], estuaries [22,23] and marine plankton [24,25], as well as in terrestrial hot springs and hydrothermal vent systems [26–29]. The shear numbers of these organisms in so many environments...
and their phylogenetic breadth indicate that they constitute a large and ubiquitous group, with some marine planktonic lineages among the most abundant microorganisms on the planet [24]. With the capacity to oxidize ammonia to nitrite and to fix CO₂, at least some of these organisms (perhaps even most of them) are now thought to be significant for global nitrogen and carbon cycles. A pure culture of *Nitrosopumilus maritimus* from the marine group I.1a has allowed for detailed physiological analysis of an AOA [30] and for sequencing of another AOA genome [31]. Furthermore, the draft genome of *Nitrososphaera gargensis*, a moderately thermophilic ammonia oxidizer growing in stable laboratory enrichments [32], has recently been obtained (GenBank accession numbers GU797788–GU797828 and HM229997–HM230053). As revealed by phylogenetic analyses of its 16S rRNA and the A subunit of ammonia monoxygenase (Amo), which catalyses the first step in the oxidation of ammonia to nitrite, this organism is closely related to the major group of AOA detected in soils (group I.1b) [19]. It thus represents a distinct group that forms a sister lineage to the marine cluster (group I.1a), which includes *N. maritimus* and *C. symbiosum*. The availability of three AOA genome sequences (Table 1) representing two different lineages facilitated a more comprehensive test of the Thaumarchaeota hypothesis and a search for common patterns in the composition of their genomes.

An updated phylogenetic analysis of the data set used by Brochier et al. (53 concatenated ribosomal proteins) [12] confirms that Thaumarchaeota are a lineage that is distinct from Euryarchaeota and Crenarchaeota, with bootstrap values (BV) of 100% and 98% supporting the monophyly of Crenarchaeota and Euryarchaeota, respectively (Figure 1c). Within Euryarchaeota and Crenarchaeota, the monophyly of all archaeal orders is robustly supported (BV >95%) and their relationships are in agreement with previous work [33] suggesting that the tree is not affected by major artefacts. Concerning the phylogenetic position of Thaumarchaeota, a basal branching is observed as in previous analyses, but with slightly lower support (BV 87%) [12]. This indicates that additional data are required for better resolution of the position of Thaumarchaeota within the archaeal domain. However, this does not alter the conclusion that Thaumarchaeota represent a distinct major archaeal lineage because their emergence within either the Crenarchaeota or the Euryarchaeota was never observed in any phylogenetic analyses (see below).

### A distinct set of information-processing genes in AOA

The presence and absence of genes involved in central informational processes (such as replication, transcription and translation) and genes involved in cell division were compared in all available archaeal genomes to identify phylogeny-specific distribution patterns. The recently published archaeal clusters of orthologous genes (arCOGs) [34] based on 41 archaeal genomes (13 crenarchaeotes including *C. symbiosum*, 27 euryarchaeotes and *Nanoarchaeum equitans*) were also considered. Several information-processing genes whose presence or absence is characteristic for Euryarchaeota and/or Crenarchaeota show a pattern in the three investigated thaumarchaeotal genomes that is distinctive from either of the two described phyla (Table 2), which points to fundamental differences in cellular processes. Thus, this gene content comparison strongly supports the Thaumarchaeota proposal, as earlier indicated on the basis of the *C. symbiosum* genome [12]. Similarly, *K. cryptophilum* exhibits a unique distribution pattern of information-processing genes. However, additional genome sequences of organisms related to *K. cryptophilum* are required before general conclusions on this provisional phylum can be made.

In the following sections we summarize and discuss the specific – and what we consider most relevant and informative – aspects of information-processing machineries in Thaumarchaeota that distinguish them from other archaeal phyla.

### Translation: Thaumarchaeota have a specific set of ribosomal proteins

All domains of life share 34 [35] ribosomal (r-) protein families, whereas Archaea and Eukarya have another 33 families in common (see below) [35]. By contrast, neither Archaea nor Eukarya share an r-protein family exclusively with Bacteria. In addition to the 34 universal r-proteins, Bacteria contain 23 domain-specific families. Eukarya encode 11 domain-specific families, whereas only the r-protein family LXA is exclusively found in Archaea [35,36]. Based on these observations it was suggested that the archaeal ribosome represents a simplified version of the

<table>
<thead>
<tr>
<th>Nitrososphaera gargensis</th>
<th>Cenarchaeum symbiosum A</th>
<th>Nitrosopumilus maritimus SCM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Habitat</td>
<td>Garga hot spring, enriched at 46°C</td>
<td>Symbiont of the marine sponge Axinella mexicana</td>
</tr>
<tr>
<td>Metabolism</td>
<td>Autotrophic ammonia oxidizer</td>
<td>Putative ammonia oxidizer</td>
</tr>
<tr>
<td>Genome size</td>
<td>&gt;2.6 Mb (draft genome*)</td>
<td>2.05 Mb</td>
</tr>
<tr>
<td>GC content</td>
<td>48%</td>
<td>57.7%</td>
</tr>
<tr>
<td>Predicted genes</td>
<td>&gt;3200*</td>
<td>2066*</td>
</tr>
<tr>
<td>Ribosomal RNA</td>
<td>1× 16S-23S rRNA operon; 1× 5S rRNA</td>
<td>1× 16S-23S rRNA operon; 1× 5S rRNA</td>
</tr>
<tr>
<td>Phylogenetic affiliation</td>
<td>Group I.1b AOA (soil group)</td>
<td>Group I.1a AOA (marine group)</td>
</tr>
</tbody>
</table>

*Evidence of a non-redundant and almost complete draft genome sequence (two gaps left at the time of paper submission) of *N. gargensis* includes the presence of all rRNA synthetases, all r-proteins (found in the genomes of the closely related *C. symbiosum* and *N. maritimus*) and all RNA polymerase subunits in one gene copy. In addition, *N. gargensis* contains a comparable number of archaeal core-COGs as found in the genomes of other Archaea (e.g. 210 *C. symbiosum*, 211 *N. maritimus*, and 215 *N. gargensis*). GenBank accession numbers of the *N. gargensis* genes discussed are GU797786–GU797828 and HM22999-HM230053.

*According to the automated annotation number (ORFs >150 bp).

Table 2. Phylum-specific genes involved in archaeal central information-processing machineries

<table>
<thead>
<tr>
<th>Ribosomal proteins</th>
<th>COG</th>
<th>Thaumarchaeota</th>
<th>Euryarchaeota</th>
<th>Nanoarchaeota</th>
<th>Crenarchaeota</th>
<th>Korarchaeota</th>
</tr>
</thead>
<tbody>
<tr>
<td>r-protein S25e</td>
<td>4901</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>r-protein S26e</td>
<td>4830</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>r-protein S30e</td>
<td>4919</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>r-protein L13e</td>
<td>4352</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>r-protein L14e</td>
<td>2163</td>
<td>-</td>
<td>-</td>
<td>+ (some)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>r-protein L34e</td>
<td>2174</td>
<td>-</td>
<td>-</td>
<td>+ (some)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>r-protein L38e</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ f -</td>
<td>-</td>
</tr>
<tr>
<td>r-protein L29p</td>
<td>0255</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>r-protein L9a</td>
<td>2157</td>
<td>-</td>
<td>-</td>
<td>+ (most)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Translation**

<table>
<thead>
<tr>
<th>Gene</th>
<th>COG</th>
<th>Thaumarchaeota</th>
<th>Euryarchaeota</th>
<th>Nanoarchaeota</th>
<th>Crenarchaeota</th>
<th>Korarchaeota</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA pol G (=rpoB)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RNA pol A – 1 ORF</td>
<td>0086</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>split</td>
<td>split</td>
</tr>
<tr>
<td>RNA pol B – 1 ORF</td>
<td>0085</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>most split</td>
<td>split</td>
</tr>
<tr>
<td>Rpo34</td>
<td>5111</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MBF1</td>
<td>1813</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EIF1</td>
<td>4888</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Replication**

<table>
<thead>
<tr>
<th>Gene</th>
<th>COG</th>
<th>Thaumarchaeota</th>
<th>Euryarchaeota</th>
<th>Nanoarchaeota</th>
<th>Crenarchaeota</th>
<th>Korarchaeota</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA pol D (small)</td>
<td>1311</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNA pol D (large)</td>
<td>1933</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RPA (Eury-like)</td>
<td>(1599)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>- (not T. p.) +</td>
</tr>
<tr>
<td>RPA / SSB (Cren-like, single OB-fold)</td>
<td>1599</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(some)</td>
<td>-</td>
</tr>
<tr>
<td>&gt; 1 PCNA homolog</td>
<td>0592</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>(not T. p.)</td>
</tr>
</tbody>
</table>

**Topoisomerases**

<table>
<thead>
<tr>
<th>Gene</th>
<th>COG</th>
<th>Thaumarchaeota</th>
<th>Euryarchaeota</th>
<th>Nanoarchaeota</th>
<th>Crenarchaeota</th>
<th>Korarchaeota</th>
</tr>
</thead>
<tbody>
<tr>
<td>Topo IB</td>
<td>3569</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Topo IA</td>
<td>0550</td>
<td>- CTD</td>
<td>- CTD</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reverse gyrase</td>
<td>1110</td>
<td>-</td>
<td>-</td>
<td>+ (HT)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Topo II A (SU A+B)</td>
<td>0187/8</td>
<td>-</td>
<td>-</td>
<td>+ (17 org.)</td>
<td>-</td>
<td>-</td>
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</table>

**Cell Division**

<table>
<thead>
<tr>
<th>Gene</th>
<th>COG</th>
<th>Thaumarchaeota</th>
<th>Euryarchaeota</th>
<th>Nanoarchaeota</th>
<th>Crenarchaeota</th>
<th>Korarchaeota</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESCRT-III (CdvB)</td>
<td>5491</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+ CdvABC b</td>
</tr>
<tr>
<td>Vps4 (CdvC)</td>
<td>0464</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+ CdvABC b</td>
</tr>
<tr>
<td>CdvA</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+ CdvABC b</td>
</tr>
<tr>
<td>Fts Z</td>
<td>0206</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Smc, Chromosome segregation ATPase</td>
<td>1196</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ScpA + ScpB</td>
<td>1354+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(many)</td>
<td>-</td>
</tr>
</tbody>
</table>

**Histones**

<table>
<thead>
<tr>
<th>Gene</th>
<th>COG</th>
<th>Thaumarchaeota</th>
<th>Euryarchaeota</th>
<th>Nanoarchaeota</th>
<th>Crenarchaeota</th>
<th>Korarchaeota</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histones (H3/H4)</td>
<td>2036</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>(exc. two) +</td>
</tr>
</tbody>
</table>

**Repair**

<table>
<thead>
<tr>
<th>Gene</th>
<th>COG</th>
<th>Thaumarchaeota</th>
<th>Euryarchaeota</th>
<th>Nanoarchaeota</th>
<th>Crenarchaeota</th>
<th>Korarchaeota</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hef nuclease</td>
<td>1111+</td>
<td>1948</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>ERCC4-type nuclease</td>
<td>1948</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>ERCC4-type helicase</td>
<td>1111</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RadB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>HSP70/DnaK, GrpE, Hsp40/DnaJ</td>
<td>0443, 0576, 0326</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hsp90-like domain</td>
<td>0326</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>UvrABC</td>
<td>0178, 0556, 0322</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(mesophiles)</td>
<td>-</td>
</tr>
</tbody>
</table>

*Abbreviations: +, present; (+), potentially present; –, absent; CTD, C-terminal domain; HT, hyperthermophiles; exc, except for; Eury, Euryarchaeota; Cren, Crenarchaeota; org, organisms; T. p., Thermophilum pendens; C. sym., Cenarchaeum symbiosum; N. mar., Nitrosopumilus maritimus; N. gar., Nitrososphaera gargensis.

**GenBank accession numbers for the N. gargensis genes discussed are GU797788–GU797828.

**cdvABC operon not present in Thermoproteales.**
eukaryotic one. In contrast to Bacteria and Eukarya, which acquired further r-proteins independently, it was suggested that the archaeal ribosome (apart from LXa) has undergone reductive evolution [35]. For example, the r-proteins L35ae and L41e are absent in almost all archaea. In addition, Euryarchaeota have lost at least five r-protein families, L38e, L13e, S25e, S26e and S30e. An even smaller number of r-proteins are encoded by later diverging euryarchaetes such as Halobacteriales [35]. Thaumarchaeota show a lineage-specific pattern that is clearly distinct from those of Crenarchaeota and Euryarchaeota. For example, all three AOA genomes possess the r-proteins S26e, S25e and S30e, which are absent in all euryarchaeaetes, but lack the r-proteins L14e and L34e, which are found in all crenarchaetes, K. cryptophilum and N. equitans. C. symbiosum and N. maritimus also lack a homologue of L13e, which is found in all crenarchaetes and K. cryptophilum. A putative L13e homologue was identified in the genome of N. gargsis, which might indicate that it was lost by reductive evolution only in the marine thaumarchaeotal lineage, as might be true for gene expression factor MBF1 [37], which is present in N. gargensis only. By contrast, putative homologues of the ribosomal protein S24e, which originally could not be identified in C. symbiosum [12], were detected in all Thaumarchaeota when the N. gargensis homologue was used as a query sequence (GU797793 for N. gargensis, Nmar0535, CENSYa0426). Interestingly, the Archaea-specific r-protein LXa is missing in both Thaumarchaeota and Korarchaeota. This might indicate a rather late acquisition of this protein in the archaeal domain because it is only present in Euryarchaeota and Crenarchaeota.

**Transcription: the RNA polymerase of Thaumarchaeota testifies to an ancient lineage**

In all three domains of life, DNA-dependent RNA polymerases (RNAPs) are composed of several subunits. Whereas the bacterial enzymes contain only five subunits, each of the three distinct eukaryotic RNAPs (I–III) consists of at least 12 subunits [38]. Archaea contain a single polymerase with 10–14 subunits, of which most are homologues of subunits of the eukaryotic polymerases [39]. Even subunit 8, until recently considered to be specific for Eukarya, is present in Crenarchaeota and Korarchaeota [40,41]. In addition, a homologue of the loosely associated eukaryotic RNAPIII-specific subunit 34 (rpc34) was detected in Crenarchaeota, Thaumarchaeota and late-diverging euryarchaeotal orders such as Archaeoglobales, Methanomicrobiales and Halobacteriales [42]. This suggests the presence of a rather complex RNAP in the last common ancestor of Archaea and Eukarya and represents a well-known example of the close relationship of information-processing machineries in these two domains of life [43]. Interestingly, in contrast to Bacteria

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**Figure 2.** Phylogenetic tree based on archaeal RpoA proteins and schematic gene cluster encoding RNA polymerase subunits A, B and H. Bayesian inference phylogeny of RpoA proteins was performed after concatenation of crenarchaeotal and euryarchaeal split archaeal RpoA subunits and alignment with the unsplit RpoA proteins of Thaumarchaeota and Korarchaeota and bacterial and eukaryotic homologues as outgroups (1051 amino acid positions, 30% conservation filter). In agreement with the unsplit rpoA genes, the phylogenetic analysis suggests early divergence of Korarchaeota and Thaumarchaeota, both of which are highly supported as distinct lineages. The red star indicates a potential split in the ancestral rpoA gene; green stars indicate potential splits in the rpoB gene; an alternative possibility is indicated by the blue star and blue circle, denoting potential split and fusion events for the rpoB gene, respectively. The scale bar represents 20% estimated sequence divergence.
Chapter III

and Eukaryota, which contain a single gene for RNAP subunit A (rpoA), in both Crenarchaeota and Euryarchaeota the A subunit is encoded by two separate genes that occur in a gene cluster encoding subunits H, B and A' and A'' [44]. Protein sequence alignments have shown that the ends of A' and the starts of A'' subunits coincide [43]. By contrast, investigation of this gene locus in K. cryptophillum [9] and Thaumarchaeota [15,38] revealed only a single rpoA gene.

Thus, the most parsimonious explanation for the split in subunit A would be that it occurred only once in evolution. This is supported by RpoA phylogeny, whereby Thaumarchaeota and Korarchaeota form two distinct lineages that branch off before Euryarchaeota and Crenarchaeota, possibly predating the rpoA split (Figure 2).

Replication and DNA-binding proteins: Thaumarchaeota are distinct from Crenarchaeota

Like Euryarchaeota and Korarchaeota, the three thaumarchaeotes contain two different archaeal DNA polymerase families (PolB and PolD). This is in contrast to Crenarchaeota, which only encode polymerases of the B family, of which all archaea contain at least one homologue (often more) [34,45]. Phylogenetic investigation of the large and small subunits of PolD revealed two monophyletic groups consisting of Euryarchaeota and Thaumarchaeota and a separated korarchaeotal branch (Figure 3), indicating early diversification of this gene in the archaeal domain and its potential loss in the lineage leading to Crenarchaeota.

Proliferating cell nuclear antigen (PCNA) is the archaeal sliding clamp involved in DNA replication [46]. Whereas only one homologue of this protein is found in Euryarchaeota, Korarchaeota and Thaumarchaeota, all Crenarchaeota (with one exception) contain two or three copies of this gene, which might be because of early gene duplication events in the respective lineages (Figure 4). Interestingly, PCNA proteins of Thaumarchaeota form a monophyletic and early-diverging archaeal group in phylogenetic analyses when eukaryotic sequences are used as an outgroup (Figure 4), whereas the korarchaeotal PCNA protein does not form a distinct lineage, indicating that it might have been acquired laterally.

Chromatin proteins with a histone fold related to eukaryotic histones H3 and H4 are encoded by most euryarchaeotes [47], except for Thermoplasmatales [48], and were long considered to be absent in Crenarchaeota. Their finding in the recently obtained genomes of K. cryptophillum and the crenarchaeotes Thermoplasmatales and Caldivirga maquilensis [47], as well as in environmental sequences and Thaumarchaeota [49], supports the previously proposed idea that histones were present in the last common ancestor of Archaea and Eukarya [47].

Figure 3. Phylogeny of archael polymerase D. Bayesian inference phylogeny of (a) the large subunit of DNA polymerase D (scale bar indicates 10% estimated sequence divergence) and (b) the small subunit of DNA polymerase D (scale bar indicates 20% estimated sequence divergence). Calculations were based on 944 and 418 aligned amino acid positions (30% conservation filter) for the large and small subunits, respectively. Numbers represent Bayesian likelihood values. As only archaea contain the complete homologue of the large polymerase D, no outgroup could be included in (a).
Topoisomerases: more surprises from Thaumarchaeota

DNA topoisomerases play a crucial role in many cellular processes that involve DNA because they solve topological problems of circular and/or large linear double helices that arise during replication, transcription, recombination and chromosome segregation [50]. Even though topoisomerases of diverse families are known to have been transferred horizontally several times in evolution, they might still serve as important genomic marker genes defining specific phylogenetic lineages [51]. Interestingly, Thaumarchaeota contain genes for topoisomerase IB, which is absent from the genomes of all other archaea described but occurs in Bacteria, Eukarya and some eukaryotic DNA viruses (e.g. mimivirus) [52]. Phylogenetic analysis of topoisomerase IB (with C. symbiosum included) showed a clear separation of two deeply branching monophyletic lineages, namely a eukaryotic–archaeal and a viral–bacterial one [52]. Within the eukaryotic–archaeal branch, topoisomerase IB of Thaumarchaeota (including sequences derived from recently published archaeal soil fosmids [53]) forms a monophyletic lineage with high bootstrap support that represents a sister group to the eukaryotic homologues [52]. Thus, topoisomerase IB does not seem to have been acquired recently by the Thaumarchaeota via lateral gene transfer from Eukarya, but rather might have already been present in the last common ancestor of archaea (LACA). According to this scenario, Thaumarchaeota would have retained topoisomerase IB during evolution, whereas all other archaea have lost this enzyme but retained the functionally related topoisomerase IA.

The genome of N. gargensis is particularly informative with respect to the potential ancestral distribution of topoisomerases. It is the only known archaeal genome that encodes both topoisomerase IA and topoisomerase IB. The topoisomerase IA gene from N. gargensis is most closely related to a homologue in the soil fosmid clone 54d9 [54].
This fosmid originated, according to a linked 16S rRNA gene, from the AOA soil group I.1b to which *N. gargensis* also belongs. Furthermore, the presence of a C-terminal domain of topoisomerase IA in both *C. symbiosum* and *N. maritimus* strengthens the hypothesis that the common ancestor of Thaumarchaeota contained topoisomerase IA as well as topoisomerase IB.

Reverse gyrase (TopoII) is a hallmark of hyperthermophilic and extremely thermophilic organisms [55], even though it has been detected in some thermophilic bacteria [56], but its absence in the moderate thaumarchaeota is not surprising and might represent an environmental rather than a phylogenetic signal. Environmental selection might also account for the presence of the UvrABC repair system and the chaperon GroEL in Thaumarchaeota and their absence in most crenarchaeota and hyperthermophilic euryarchaeota [57]. In this respect, the genome of the first enriched thermophilic ammonia-oxidizer *Nitrosocaldus yellowstonii* [26] will be of special interest, because this might help to distinguish genes that were vertically inherited from those acquired by lateral gene transfer or lost in the course of adaptation to high or mesophilic temperatures, respectively.

**Cell division: genes for two modes of cell division in Thaumarchaeota**

The tubulin-related GTPase FtsZ represents the major cell-division protein in most bacteria and in Euryarchaeota [58]. Crenarchaeota, however, do not encode FtsZ proteins, but seem to exploit a different mode of cell division [59–60] that involves three proteins, termed CdvA, CdvB and CdvC [59]. CdvA is a protein with a coiled-coil domain, CdvB is homologous to an endosomal sorting complex required for transport (ESCRT)-III like protein and CdvC is homologous to the AAA+type ATPase Vps4 [60]. The homologous proteins in Eukarya are involved in multivesicular body formation, cell division and virus budding [61–63]. The proposed crenarchaeotal cell division proteins (CdvA–C) occur in an operon in all sequenced members of Sulfolobales and Desulfothermococcales, whereas they are absent in Korarchaeota and Euryarchaeota [59]. Interestingly, organisms of the crenarchaeotal order Thermoproteales encode neither of these novel cell-division proteins nor FtsZ. Rather, the three thaumarchaeotes contain homologues of both systems [59–61] and might thus reflect the ancestral distribution of those genes in the LACA. Even though homologues of the three newly identified crenarchaeotal cell-division genes (*cdvA*, *cdvB*, *cdvC*) are present in Thaumarchaeota, they do not occur as clusters in the genomes but are rather randomly distributed. In a phylogenetic analysis of CdvA, Thaumarchaeota form a monophyletic and only distantly related sister group to Crenarchaeota (Figure 5), suggesting that the presence of *cdvA* in these two phyla does not result from a recent lateral gene transfer. Furthermore, the CdvB/ESCRT-III-encoding genes of Thaumarchaeota and Crenarchaeota differ. In Crenarchaeota, several CdvB/ESCRT-III-encoding genes are usually present and one of these orthologues is always associated with the CdvABC operon. This operon-associated *cdvB* gene has a conserved C-terminal extension containing a winged-helix domain (~50 aa in length), which is missing in the other CdvB homologues [60]. None of the three *cdvB* orthologues in *N. maritimus* and *C. symbiosum* or the five *cdvB* orthologues in *N. gargensis* contains this C-terminal domain.

Thaumarchaeota encode homologues of both archaeal cell-division systems (from Euryarchaeota and Crenarchaeota), so it remains to be shown experimentally which system is actually used during growth or whether proteins of both cell-division machineries interact. Such analyses might be particularly fruitful because they could shed light on the mode of cell division in LACA and potentially on the evolution of cell division mechanisms in Eukarya [64].

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**Figure 5.** Bayesian inference phylogeny of the archaean cell division protein CdvA. A 30% conservation filter was used. Calculation was stopped after 50 000 generations because a clear dichotomy between Crenarchaeota and Thaumarchaeota was apparent. The scale bar refers to 10% estimated sequence divergence. Numbers at nodes represent Bayesian likelihood values.
Conclusions
Phylogenetic analyses of several different marker genes and comparative gene content analysis of the genomes of three AOA suggests that they are indeed clearly distinct from all other archaea. From an evolutionary point of view, they form a separate and frequently deep-branching lineage within the domain Archaea. Although the exact placement of this lineage, in particular in relation to the Korarchaeota, cannot be unambiguously resolved before more genome sequences of both groups are available, there is no doubt that the AOA do not belong to Crenarchaeota, as initially suggested by comparative 16S rRNA sequence analysis. Consequently, these findings provide compelling support for the recently proposed assignment of the AOA to the new archaeal phylum Thaumarchaeota [12].

There are indications that Thaumarchaeota might also contain a specific membrane lipid: the tetraether lipid crenarchaeol was originally associated only with marine planktonic archaea [65], but was later found in many terrestrial hot springs [27,66–70] and was recently identified as a component of the lipid membranes of three AOA, *N. maritimus* [71], *N. yellowstonii* [26] and *N. garsengensis* [72]. No crenarchaeote has been shown to harbour crenarchaeol; it might rather represent a Thaumarchaeota-specific membrane lipid and could possibly be called thau- marchaeol instead.

The recognition of a third archaeal phylum, the Thaumarchaeota, opens new perspectives regarding our view of the evolution of archaea and early cellular life forms. For example, it allows for suggestions on the occurrence of certain features in LACA. According to the gene content of the AOA in comparison with other archaeal phyla, it seems likely that LACA contained, for example, the complete set of r-proteins that are shared with Eukarya, a protein with a histone fold, a single gene for RNA polymerase A, an ftsZ homologue as well as cdv genes, topoisomerase IA as well as topoisomerase IB, and DNA polymerase D and DNA polymerase B. This indicates that LACA was probably more complex than anticipated, as suggested in other studies [34,35,73].

Investigation of Thaumarchaeota has already led to several unexpected discoveries, including the recognition that archaea are far more abundant in moderate aerobic habitats than originally anticipated [19,24] and that they contribute to nitrification [74–76] and exhibit extraordinary physiological properties [77]. Genomic and biochemical analyses indicate that these organisms almost certainly harbour even more wonders [78,79] that will be fully revealed only when their biochemical and cellular features are characterized in more detail.

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Chapter IV

Novel group I.1a ammonia-oxidizing archaea are globally distributed in thermal habitats: enrichment and genome analysis of the first representative, “Candidatus Nitrosotenuis uzonensis”

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Novel group I.1a ammonia-oxidizing archaea are globally distributed in thermal habitats: enrichment and genome analysis of the first representative, “Candidatus Nitrosotenuis uzonensis”

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Summary

The recent discovery of ammonia-oxidizing archaea (AOA) and the detected high abundance of AOA in many aquatic and terrestrial habitats have dramatically influenced our understanding of nitrifying microbial communities. Moreover, AOA are the only aerobic ammonia oxidizers known to be active in geothermal environments. Besides previously described thermophilic AOA, a phylogenetic lineage within the “marine” group I.1a of *Thaumarchaeota* appears to be globally distributed in terrestrial high-temperature habitats based on molecular data. These organisms might play crucial roles in thermal ecosystems, but have not been functionally characterized yet. Here we report on the enrichment and genomic characterization of a moderately thermophilic representative of this lineage from a hot spring in Kamchatka. This thaumarchaeote, provisionally classified as “*Candidatus Nitrosotenuis uzonensis*”, is a chemolithoautotrophic ammonia oxidizer. The nearly complete genome sequence of the novel archaean provides initial insights into the pathways used for ammonia oxidation and carbon fixation. Genome comparisons to other AOA revealed a substantial degree of genomic plasticity with unique genomic regions in “*Ca. N. uzonensis*”, which likely are determinants of ecological niche differentiation.
Introduction

The discovery of ammonia-oxidizing archaea (AOA) (Könneke et al., 2005) fundamentally revised our view of nitrogen cycling in the environment and sparked a hitherto unprecedented wave of studies related to the importance of archaea in moderate ecosystems. It culminated in the proposal of a third archaeal phylum named *Thaumarchaeota* (Brochier-Armanet et al., 2008; Spang et al., 2010), whose characterized representatives share ammonia oxidation as their only demonstrated pathway for energy conservation. Today, there is compelling evidence for a major role of AOA in the first step of nitrification, the aerobic oxidation of ammonia to nitrite (Prosser and Nicol, 2008; Erguder et al., 2009; Schleper and Nicol, 2011). The importance of AOA in other biogeochemical conversions, namely denitrification (Treusch et al., 2005; Bartossek et al., 2010) and carbon cycling (Damsté et al., 2002b; Könneke et al., 2005; Hallam et al., 2006b; Hallam et al., 2006a; de la Torre et al., 2008; Hatzenpichler et al., 2008; Walker et al., 2010), is under debate.

Past research on nitrification mainly focused on ammonia-oxidizing bacteria (AOB), which are known to thrive in most terrestrial and aquatic ecosystems (Koops and Pommerening-Röser, 2001; Koops et al., 2003). Meanwhile, however, AOA have been shown to occur and outnumber AOB in many environments, including soil (Leininger et al., 2006; He et al., 2007; Adair and Schwartz, 2008; Shen et al., 2008), oceanic waters (Wuchter et al., 2006; Nakagawa et al., 2007; Beman et al., 2008) and sediments (Caffrey et al., 2007; Park et al., 2008). The environmental factors shaping the community structures of AOA and AOB are highly debated. Low pH values, the presence of sulphide, and low substrate concentrations have been postulated to select for AOA in the environment (reviewed by Erguder et al., 2009). A recent study demonstrated that “*Candidatus Nitrosopumilus maritimus*”, which represents a group of AOA globally distributed in marine ecosystems (Francis et al., 2005; Könneke et al., 2005; Wuchter et al., 2006; Labrenz et al., 2010), exhibits remarkable adaptations to ammonia limitation, such as an extremely high affinity to ammonia (Martens-Habbena et al., 2009). These physiological traits may explain the ubiquity of AOA in the nutrient-deprived open oceans (Francis et al., 2005; Könneke et al., 2005; Wuchter et al., 2006; Labrenz et al., 2010) and raise important questions on the validity of current biogeochemical models (Martens-Habbena et al., 2009).

In contrast to AOB, which so far have not been convincingly detected in thermal environments, AOA also play a crucial role for nitrification in these extreme habitats (de la Torre et al., 2008; Reigstad et al., 2008; Jiang et al., 2010). Enrichment cultures of thermophilic (74 °C) (de la Torre et al., 2008) and moderately thermophilic (46 °C)
AOA have become available, and the global presence of AOA in high temperature habitats up to 94 °C has been demonstrated. So far, gene sequences indicative for AOA have been found in subsurface thermal springs (Spear et al., 2007; Weidler et al., 2008), many terrestrial hot springs (Reigstad et al., 2008; Zhang et al., 2008; Jiang et al., 2010; Zhao et al., 2011) and deep-sea hydrothermal vents (Wang et al., 2009a; Wang et al., 2009b). Measurements of in situ nitrification (Reigstad et al., 2008) and of ammonia monooxygenase subunit alpha (amoA) gene transcription (Jiang et al., 2010) in several hot springs further indicate an important role of thermophilic AOA in these systems.

The presence of the lipid crenarchaeol in terrestrial hot springs (Pearson et al., 2004; Schouten et al., 2007; Pearson et al., 2008; Pitcher et al., 2009a) lends additional support to the importance of AOA in thermal habitats. This membrane lipid was originally thought to be specific for mesophilic crenarchaeotes (Damsté et al., 2002b; Damsté et al., 2002a), but now is known to be an important lipid component of both moderate (Schouten et al., 2008; Pitcher et al., 2011) and thermophilic AOA (de la Torre et al., 2008; Pitcher et al., 2009b). Recently, the new name thaumarchaeol was suggested for this lipid, which appears to be a specific marker for the newly proposed phylum Thaumarchaeota (Spang et al., 2010).

Despite the global distribution and obvious ecological significance of AOA, insights into the physiology and genomic make-up of these organisms are still sparse. This knowledge gap can partly be attributed to the difficulties of cultivating the fastidious and slow-growing AOA under laboratory conditions. Only very few enrichment or pure cultures are available, which are difficult to maintain, and the enrichment and isolation of new AOA-strains is a time-consuming and tedious procedure.

In this study, we inoculated enrichment media with samples from a hot spring located in the Uzon caldera on the Kamchatka peninsula (Russia). Stable ammonia-oxidizing enrichment cultures were established and grown at 46 °C. We present evidence that in one of these cultures a novel, moderately thermophilic AOA strain is solely responsible for the observed oxidation of ammonia. We demonstrate that this thaumarchaeote, which we designate as “Candidatus Nitrosotenuis uzonensis”, represents a lineage of AOA that is globally distributed in terrestrial high temperature environments but was not brought into culture previously. Furthermore, we present preliminary insights gained from analyzing the near-complete genome sequence of this AOA.
Results and discussion

An ammonia-oxidizing enrichment culture (Fig. 1) was established and maintained in the laboratory for four years. During the initial enrichment phase, different incubation temperatures were tested. Based on these tests, 46 °C was chosen for all further incubations, because this temperature resulted in the highest nitrite production rates (data not shown). In later stages of the enrichment nitrite accumulated (Fig. 1), indicating the absence of active nitrite-oxidizing bacteria (NOB). Recently, a parallel enrichment of NOB from the same thermal spring resulted in the successful cultivation of a member of the phylum *Nitrospira* (Lebedeva et al., 2011). Below we refer to our ammonia-oxidizing enrichment as “culture N4”.

Screening for ammonia-oxidizing bacteria

Several molecular methods were applied to screen culture N4 for the presence of known AOB. Bacterial 16S rRNA genes were PCR-amplified from the enrichment, cloned, sequenced, and subjected to phylogenetic analysis. Among 30 screened bacterial 16S rRNA clones, none showed affiliation to AOB (data not shown). Consistent with these results, all attempts to PCR-amplify bacterial *amoA* genes from this culture were unsuccessful. No AOB were detected by fluorescence *in situ* hybridization (FISH) using established rRNA-targeted probes that are specific for all known groups of betaproteobacterial AOB (Tab. S1). This is consistent with the absence of gene sequences indicative for AOB in the obtained metagenomic dataset (see below). A newly discovered betaproteobacterium, which represents a novel genus and was present in several of our AOA enrichments (Hatzenpichler et al., 2008) and unpublished data), was not detectable by FISH (Tab. S1) in the late stages of culture N4, but was present at the time of sampling for metagenome sequencing.

Molecular detection of a novel ammonia-oxidizing thaumarchaeote

During the early stages of enrichment N4 an archaeal 16S rRNA gene library was established and eight clones were randomly selected and sequenced. At a later stage of the culture, a second 16S rRNA gene library was established. Screening of 31 clones from this library by restriction fragment length polymorphism (RFLP) analysis yielded three band patterns, which were represented by ten nearly full-length 16S rRNA sequences. All sequences obtained from these two libraries were highly similar to each other (> 99.0%), fell within the group I.1a *Thaumarchaeota*, and were only distantly related to cultured AOA with “*Ca. N. maritimus*” as the closest cultured relative (92.8% similarity). Similar 16S rRNA sequences have only
rarely been obtained from the environment and most of them originate from thermal habitats, such as subsurface geothermal water (closest hit: AB113625, 99.8% similarity), or soil (Fig. 2a).

Clone libraries were also established for archaeal amoA and amoB (ammonia monooxygenase subunits alpha and beta) genes, and 39 and 12 clones were randomly picked and sequenced, respectively. All obtained amoA and amoB gene sequences were highly similar (each ≥ 99.1% nucleotide similarity) to each other and differed clearly from those of other cultured AOA (Fig. 2bc and Fig. S2).

Furthermore, we established a clone library of the gene hcd coding for archaeal 4-hydroxybutyryl-CoA dehydratase, which is a key enzyme of the 3-hydroxypropionate/4-hydroxybutyrate cycle for autotrophic CO₂ fixation (Berg et al., 2007). RFLP analysis of 25 hcd clones revealed two different band patterns, and six clones representing either pattern were sequenced and phylogenetically analyzed (Fig. 2d). The sequences were highly similar (≥ 99.5% nucleotide similarity) to each other, but showed only moderate similarity to thaumarchaeotal hcd sequences from other environments and cultures. Primers recently developed for the detection of archaeal hcd (Offre et al., 2011) and accA (Yakimov et al., 2009) genes in marine and soil samples contain up to ten nucleotide mismatches to the respective gene sequences of the novel thaumarchaeote and of “Candidatus Nitrososphaera gargensis” (Hatzenpichler et al., 2008). For example, the hcd-specific primers (Offre et al., 2011) had one central and one 3’ lateral mismatch (forward primer) and one central mismatch (reverse primer) to the hcd sequence obtained later in this study by metagenomics. PCR-amplification of the hcd gene fragments was successful despite these mismatches, but these primer sets may not be generally applicable to detect their target genes in AOA closely related to the novel thaumarchaeote or “Ca. Nitrososphaera gargensis”.

Taken together, the analyses of four molecular marker genes demonstrated that culture N4 contained a novel ammonia-oxidizing thaumarchaeote that is only distantly related to other cultured AOA.

**Genome reconstruction of the novel archaeon**

The nearly complete genome of the novel, moderately thermophilic AOA was reconstructed using an environmental genomics approach (for details please refer to the Experimental procedures section). At the time of sampling for genome sequencing, thaumarchaeotal cells constituted ~50% of the organisms in culture N4 according to visual quantification after FISH with catalyzed reporter deposition (CARD-FISH).
The genome of the novel AOA is represented by a single scaffold containing 14 contigs with a total length of 1,649,125 bp, a mean sequence coverage of 39.4 and an average GC-content of 42.2%. Other key features of the genome are listed in Tab. 1 and Fig. S1. The near completeness of the genome is indicated by the presence of all 138 clusters of orthologous groups (COGs) of proteins that are encoded in all sequenced archaeal genomes (archCOGs; data not shown). The 16S rRNA, amoA, amoB, and hcd gene sequences in the genome were highly similar (>99% for each gene) to the sequences retrieved from the aforementioned clone libraries, demonstrating that the sequenced genome belongs to the same lineage as the AOA previously detected in culture N4. No gene sequences indicative for the presence of another AOA-species or known AOB or NOB were found in the obtained metagenome.

Ammonia oxidation biochemistry

The genome of the enriched thaumarchaeote encodes all proteins (Tab. S4) that recently have been proposed to be involved in the oxidation of ammonia and subsequent electron transport in “Ca. N. maritimus” (Walker et al., 2010). Considering the distant phylogenetic relationship and different habitat of the novel thaumarchaeote compared to other AOA, the conservation of this protein set in the novel and previously available genomes (Walker et al., 2010; Blainey et al., 2011) further supports the proposed key functional role in the metabolism of these organisms. The biochemistry of ammonia oxidation in AOA appears to be fundamentally different from the pathway used by known AOB and is far from being understood (Walker et al., 2010; Schleper and Nicol, 2011). A striking feature of all available AOA genomes is the lack of hydroxylamine oxidoreductase (Hao), which is a key enzyme for ammonia oxidation in AOB. The high numbers of copper-containing proteins in AOA (Hallam et al., 2006a; Walker et al., 2010; Blainey et al., 2011; Schleper and Nicol, 2011) (Tab. S4), together with the lack of c-type cytochromes, suggest a different electron transport mechanism (Walker et al., 2010; Schleper and Nicol, 2011) than found in the highly iron-dependent AOB (Wei et al., 2006; Godfrey and Glass, 2011). Recently, the reaction product of archaeal Amo was proposed to be nitroxyld hydride (HNO), which is further oxidized to nitrite via a nitroxyld oxidoreductase (Nxor) (Walker et al., 2010). Hypothetically, the activation of oxygen might also be achieved by nitric oxide (NO) produced by nitrite reductase (NirK) (Schleper and Nicol, 2011; Klotz, unpublished). Genes coding for NirK exist in “Ca. N. maritimus” and “Candidatus Nitrosoarchaeum limnia” (Walker et al., 2010; Blainey et al., 2011) as well as in the AOA presented here, but are absent from the genome of “Ca. C. symbiosum” (Bartossek et al., 2010). Interestingly, the expression of Thaumarchaeota-related nitrite reductases was
recently reported to also occur under aerobic, potentially ammonia-oxidizing, conditions in soils (Bartossek et al., 2010; Schleper and Nicol, 2011), suggesting a different function of these enzymes in AOA than in their bacterial counterparts.

Like the other three genome-sequenced AOA, the analyzed thaumarchaeote possesses single copies of the \( \text{amoA} \) and \( \text{amoB} \) genes, whereas two copies of \( \text{amoC} \) are present. Intriguingly, the AmoA-sequence of the novel thaumarchaeote is closely related to sequences that were obtained from geographically distant thermal and hot terrestrial environments (Fig. 2b and Fig. S2), such as Austrian subsurface radioactive thermal springs (Weidler et al., 2007), geothermal mine waters in the United States (Spear et al., 2007), and numerous hot springs in Asia, North America, and Iceland that exhibit a wide range of physicochemical parameters (pH 3-8.4; 1.2-1,167 µM \( \text{NH}_4^+ \)) and temperatures up to 97°C (Reigstad et al., 2008; Zhang et al., 2008; Jiang et al., 2010; Zhao et al., 2011). Their global distribution in hot environments points to a crucial ecological role of these novel AOA in these habitats. Indeed, and consistent with the ammonia-oxidizing activity of the enrichment culture N4, indirect evidence suggests that the novel AOA are active ammonia oxidizers in hot springs at temperatures of 84-85°C (Reigstad et al., 2008) and 44-94°C (Jiang et al., 2010). Moreover, the membrane lipid thaumarchaeol is present in hot springs (Pearson et al., 2004; Schouten et al., 2007; Pearson et al., 2008; Pitcher et al., 2009a). So far thaumarchaeol has exclusively been found in AOA, but thaumarchaeotes that possess this lipid and do not oxidize ammonia might still be hidden in nature.

While two AmtB-type ammonium transporters were identified (Tab. S4), we found no genes coding for a urea transporter or a urease, which would enable the organism to derive energy from urea. This is consistent with the absence of such genes in “\( \text{Ca. N. maritimus} \)” and “\( \text{Ca. N. limnia} \)” (Walker et al., 2010; Blainey et al., 2011). In contrast, “\( \text{Ca. C. symbiosum} \)” was reported to harbour all essential genes for ureolysis (Hallam et al., 2006a), but experimental support for this activity is lacking so far.

**Carbon fixation**

Recent data suggest that AOA grow autotrophically by fixing inorganic carbon via a modified 3-hydroxypropionate/4-hydroxybutyrate pathway (3HP/4HB) (Hallam et al., 2006a; Berg et al., 2007; Walker et al., 2010). Two key enzymes of this cycle are 4-hydroxybutyryl-CoA dehydratase (Hcd) and acetyl-CoA/propionyl-CoA carboxylase (AccA/PccB) that recently have been used to study the distribution and abundance of autotrophic thaumarchaeotes in the environment (Zhang et al., 2010; Offre et al., 2011; Pratscher et al., 2011). The successful
amplification of archaeal hcd gene sequences from enrichment culture N4 (Fig. 2d), which contained bicarbonate/carbon dioxide as the sole source of carbon, indicated that the novel thaumarchaeote also uses this pathway for the fixation of inorganic carbon. This hypothesis was later corroborated by the presence of a near complete 3HP/4HB cycle in the genome of the enriched AOA (Tab. S3). This finding supports the proposed wide distribution of the 3HP/4HB cycle in AOA and is in agreement with the observed thermotolerance of this carbon fixation pathway (Berg, 2011). The use of different autotrophic pathways must have important consequences for the biochemistry and ecological adaptation of AOA and AOB. For example, in the 3HP/4HB cycle bicarbonate is the fixed carbon species, whereas CO₂ is the primary substrate in the Calvin cycle operating in AOB (Arp et al., 2007). At neutral and slightly alkaline pH values bicarbonate is the predominant carbon species. In addition, already a partial 3HP/4HB cycle may enable the co-assimilation of different organic compounds including fermentation products (Berg et al., 2010; Berg, 2011). Whether these attributes are important for autotrophic AOA in hot habitats, some of them living at highly acidic pH values, is yet unknown and may be clarified in future research. Moreover, the 3HP/4HB cycle requires fewer cofactors than the Calvin cycle (Berg et al., 2010; Berg, 2011), whose high demand for cofactors would probably not be compatible with the very oligotrophic conditions that favour AOA (Martens-Habbena et al., 2009).

**Genome plasticity**

The obtained genome sequence was compared to the genomes of “Ca. Cenarchaeum symbiosum” and of “Ca. N. maritimus” to search for regions of genomic plasticity (RGP) that mainly consist of genes specific for the novel thaumarchaeote. This analysis revealed numerous RGP with a total length of ~510 kb (Fig. S1). Most genes in these regions encode hypothetical proteins or proteins of unknown function and lack homologs in the other AOA genomes, suggesting that they might be involved in habitat-specific adaptations such as life at elevated temperatures. Interestingly, the novel thaumarchaeote encodes a number of genes that have been associated with archaeal chemotaxis and flagellar assembly. These genes are located in RGP, are mostly absent from the genomes of “Ca. C. symbiosum” and “Ca. N. maritimus” (Tab. S5), but were identified in “Ca. N. limnia” (Blainey et al., 2011). Not all genes necessary for chemotaxis and motility could be identified in the genome from culture N4 and we assume that the missing genes would be located in gaps between contigs. This appears likely, because most of the identified chemotaxis and motility genes are located at contig ends directly adjacent to gaps (Tab. S5, Fig. S1) and because most of the respective
homologous genes are also clustered in a single region in the genome of “Ca. N. limnia” (Blainey et al., 2011). Their chemotaxis and motility genes most likely enable these AOA to react to changing substrate concentrations and/or to escape detrimental conditions. In contrast, these features seem to be less important in the habitats of AOA lacking such genes. In the open ocean, where “Ca. N. maritimus” resides (Francis et al., 2005; Könneke et al., 2005; Wuchter et al., 2006; Labrenz et al., 2010), water mixing is the dominant mode of microbial and nutrient dispersal and “Ca. C. symbiosum” occurs within sponge tissues that constitute a protected environment with relatively constant conditions (Preston et al., 1996).

The comparative genomic analysis between the enriched archaeon and “Ca. N. maritimus” also showed that 1,196 coding sequences (CDS) (61.08%) of the novel thaumarchaeote and 1,205 CDS (67.13%) of N. maritimus are organized in 106 syntenic regions (syntons) with an average length of 11 CDS. A genome-wide dot plot (Fig. S3a) confirmed a high degree of genomic synteny between these two AOA, which was also found in comparison to “Ca. N. limnia” (Fig. S3b). When compared to “Ca. C. symbiosum” in total 157 syntons were detected that contained 1,113 CDS (56.8%) of the novel thaumarchaeote and 1,125 CDS (55.53%) of “Ca. C. symbiosum”. However, these syntons were shorter with an average length of 7 CDS and much more scattered, with genome-scale synteny being very low (Fig. S3c). A low overall synteny to “Ca. C. symbiosum” has also been reported for the other two available genomes of AOA (Walker et al., 2010; Blainey et al., 2011), indicating that numerous genomic rearrangements must have occurred during the evolution of C. symbiosum whose lifestyle as sponge symbiont differs markedly from that of the free-living AOA.

**Provisional classification and conclusion**

Here we demonstrated that in the analysed moderately thermophilic enrichment culture N4, a group I.1a thaumarchaeote is responsible for the observed oxidation of ammonia. We propose the provisional classification of the novel archaeon as “Candidatus Nitrosotenuis uzonensis”. The name refers to the small, slender size of the rod-shaped cells (0.6±0.1 µm in length, 0.3±0.1 µm in diameter, n=20; cell morphology might be altered due to the CARD-FISH protocol applied to detect and visualize the cells) and the sampling location, a hot spring in the Uzon caldera of the Kamchatka peninsula. The archaeon is phylogenetically related to group I.1a (“marine group”) thaumarchaeotes (Brochier-Armanet et al., 2008; Spang et al., 2010), with only distant affiliation to other cultured AOA, and gains energy from the chemolithotrophic oxidation of ammonia to nitrite. In addition to N. yellowstonii of group HWCGIII (de la Torre et al., 2008) and N. gargensis representing group I.1b (Hatzenpichler
et al., 2008), this is the third identified AOA-strain growing at elevated temperatures. “Ca. N. uzonensis” is the first cultured representative of a group of AOA with worldwide distribution in terrestrial thermal habitats. Its discovery strongly corroborates the important role of this lineage for nitrogen cycling in these extreme ecosystems.

**Experimental procedures**

*Enrichment culture*

An ammonia-oxidizing enrichment culture, inoculated with a microbial mat/water sample from a terrestrial thermal spring located in the Uzon caldera on the Kamchatka peninsula (Russia), was established and maintained for four years. A temperature of 45°C and a pH of 6.5 were measured at the sampling site. The concentration of ammonium was 34.4 µM, whereas nitrite and nitrate were not detectable (Lebedeva et al., 2011). At an early stage of the enrichment, different incubation temperatures were tested (37-55 °C) in parallel to determine the optimal conditions for ammonia oxidation. The culture was grown either aerobically in 300 ml Erlenmeyer flasks or microaerobically in closed 150 ml (Wheaton Science Products, Millville, NJ, USA) or 250 ml (Schott AG, Mainz, Germany) flasks. The cells were grown in medium containing (per litre) 54.4 mg KH₂PO₄, 74.4 mg KCl, 49.3 mg MgSO₄×7H₂O, 584 mg NaCl, 33.8 µg MnSO₄, 49.4 µg H₃BO₃×7H₂O, 43.1 µg ZnSO₄×7H₂O, 37.1 µg (NH₄)₆Mo₇O₂₄×4H₂O, 97.3 mg FeSO₄×5H₂O, and 25.0 µg CuSO₄×2H₂O. Furthermore, either 147 mg CaCl₂ or 4.0 g CaCO₃ l⁻¹ (which partially precipitated) was added. At late stages of the enrichment, only CaCO₃-based medium was used. Typically, fresh medium contained 0.2-1 mM ammonium (NH₄Cl) and ~5 mM bicarbonate (NaHCO₃) at a pH of 7.4-7.8, which was adjusted using 1% (w/v) bicarbonate solution. To achieve microaerophilic conditions, only a small headspace of air was left in the closed flasks. The enrichment culture was regularly transferred into fresh medium. For this purpose, flasks containing fresh medium were inoculated with 10 vol% of the culture. The concentrations of nitrite and nitrate were regularly measured by using Merckoquant test stripes (Merck, Vienna, Austria). For recording substrate turnover, ammonium, nitrite, and nitrate concentrations were measured as recently described (Hatzenpichler et al., 2008; Bollmann et al., 2011).
Amplification, cloning, sequencing and phylogenetic analyses of 16S rRNA, amo and hcd genes

DNA was extracted from enrichment culture N4 either by phenol/chloroform-extraction including a bead-beating step (30 sec, 6 m/s; adapted from (Lueders et al., 2004)) or by using the PowerSoil kit (MOBio Laboratories, Darmstadt, Germany) according to the instructions of the manufacturer. PCR reaction mixtures contained 2 mM MgCl$_2$ and 1 U of Taq-polymerase (Fermentas Life Sciences, St. Leon-Rot, Germany) in combination with 50 pmol of the respective oligonucleotide primers. In all reactions 30 cycles were used for amplification of target DNA and a final elongation step of 10 min at 72 °C was performed. A list of all primers with the applied annealing temperatures is provided in Tab. S2. Amplification of bacterial amoA genes was attempted by using primers amoA1F and amoA2R (Rotthauwe et al., 1997), but no amplicons were obtained from the enrichment even when up to 40 PCR cycles were performed. In contrast, controls using a plasmid carrying the amoA gene of Nitrosomonas sp. as template consistently yielded positive results. Cloning and sequencing were performed as described elsewhere (Hatzenpichler et al., 2008). The cloned 16S rRNA and hcd genes were screened, prior to sequencing, by RFLP analysis using the restriction endonucleases MspI and AluI (16S rRNA) or RsaI (hcd) (Fermentas Life Sciences, St. Leon-Rot, Germany), whereas amoA and amoB clones were randomly chosen for sequencing without RFLP analysis. For each unique RFLP pattern at least one representative clone was sequenced. Phylogenetic analyses were conducted using the programs ARB (Ludwig et al., 2004) and Phylip (Felsenstein, 1993) with comprehensive databases. Automatic tools included in ARB were used to align sequences to the respective in-house databases. All alignments were then manually refined. A 16S rRNA consensus tree was reconstructed based on Maximum-Likelihood (ML) calculation (using the Hasegawa, Kishino and Yano substitution model) in which all nodes with parsimony bootstrapping (100 iterations) support below 70% were collapsed (Robertson et al., 2005). A sequence filter considering only positions conserved in ≥50% of all thaumarchaeotal and crenarchaeotal sequences in our database was used (resulting in 1,360 alignment positions for the tree calculations) and only sequences ≥1,300 nucleotides in length were considered. For the analyses of AmoA (198 considered amino acids), AmoB (152 amino acids) and Hcd (125 amino acids) proteins, Fitch-Margoliash distance trees (7 jumbles, Kimura substitution model, randomized sequence order) were reconstructed using Phylip without the use of phylogenetic filters. In addition, parsimony bootstrapping (1,000 iterations) analyses were conducted using ARB.
**FISH and CARD-FISH**

For the *in situ* detection and visualization of bacterial and archaeal cells in enrichment culture N4, FISH and CARD-FISH were performed as described elsewhere (Hatzenpichler et al., 2008) with a hybridization time of 3 h and the probes listed in Tab. S1. Fluorescein- and Cy3-labelled tyramides were synthesized according to Pernthaler et al. (Pernthaler et al., 2004) and diluted 1:1,000 in amplification buffer. After FISH or CARD-FISH, cells were stained with 4',6'-diamidino-2-phenylindole (DAPI), by using a 1 µg ml⁻¹ DAPI solution, and were microscopically analyzed using a Zeiss LSM 510 confocal laser scanning microscope and the included software. The absence of unspecific autofluorescence in the samples was confirmed by using probe NON-EUB (Tab. S1), which does not bind to any known organism.

**Metagenomic sequencing and annotation**

For obtaining biomass for metagenome sequencing, enrichment culture N4 was grown aerobically in 300 ml Erleyenmeyer flasks as described above. Biomass was harvested by centrifugation (11,000 rpm, 10 min, room temperature) and frozen (-20 °C) until phenol/chloroform-based DNA extraction (see above) was performed. The genomic DNA was submitted to mechanical fragmentation through nebulization (hydroshear) with a 3 kb mean fragment size. After recircularization using an adapter, the DNA was fragmented using an E210 ultrasound DNA-shearer (Covaris, Woburn, MA), and 600 bp fragments containing the adapter were selected for sequencing. Three quarters of a 454 Titanium mate-paired run were performed, generating ~250 Mb on 796,942 sequence reads. In addition, Illumina (GA-IIx) single read sequencing was performed, generating 34,689,904 reads of 36 bp each. The 454-reads were subjected to assembly by using the Newbler software package, generating 739 contigs on 41 scaffolds with a cumulative size of ~5.5 Mb. The largest of these scaffolds, containing 14 contigs with a total length of 1,649,125 bp and a mean contig coverage of 39.4, represents the genome of the enriched thaumarchaeote. The Illumina reads were used to correct 454 sequencing errors as described by Aury et al. (Aury et al., 2008). Corrected contigs assembled using the scaffold description were used to generate the final sequence prior annotation. The MaGe annotation platform (Vallenet et al., 2006) was used for CDS prediction, automatic annotation, and manual annotation refinement according to guidelines as recently described (Lücker et al., 2010). Statistics about syntenic regions (syntons) in compared genomes were generated by the respective tools of MaGe, where the gap parameter, which represents the maximum number of consecutive genes that are not involved in a syntton, was set to five genes. Genome-scale dot plots (Fig. S3) were generated by using the PROmer
and mummerplot programs contained in the MUMmer software package (version 3.22) (Kurtz et al., 2004) in combination with Gnuplot (version 4.4.3). The circular genome map (Fig. S1) was generated using the software Circos (version 0.54) (Krzywinski et al., 2009).

Sequence deposition
The genome sequence of “Candidatus Nitrosotenuis uzonensis” has been deposited in the GenBank database (accession number ###).

Acknowledgements
We thank Christian Baranyi, Faris Behnam and Gabriele Schwammel for technical assistance, Thomas Rattei for calculation of ArchCOGs, Valerii Fedorovich Galchenko for providing samples from a terrestrial thermal spring on the Kamchatka Peninsula (Uzon Caldera), and Anja Spang for helpful discussions. This research was funded by the Austrian Academy of Sciences via a DOC (PhD) fellowship to R.H. (2007-2009) and by the Vienna Science and Technology Fund (WWTF) via grant LS09-040 to H.D.

Figure legends
Fig.1. Near-stoichiometric oxidation of ammonia (diamonds/circles) and production of nitrite (triangles/squares) by enrichment culture N4 during an incubation period of 15 days. Two replicate culture flasks (indicated by white and black colored symbols) were inoculated with 10 vol% of the parent culture, leading to an initial nitrite concentration of ~0.5 mM. Nitrate was not detectable during the whole experiment. Axis scaling is identical for the ammonia and nitrite concentrations.

Fig. 2. Phylogenetic trees inferred from the 16S rRNA, Amo, and Hcd sequences of “Candidatus Nitrosotenuis uzonensis” and related archaea. All trees show only thaumarchaeotal group I.1a-related sequences, and the respective group I.1b-affiliated sequences were used as outgroup. Circles on tree nodes indicate high parsimony bootstrap support ≥90 %. Numbers in parentheses indicate the number of sequences within a group. Scale bars indicate 10% estimated sequence divergence. SAGM, South African Gold Mine. For an extended AmoA tree please refer to Fig. S2.
Supplementary figures legends

Fig. S1. Circular representation of the “Candidatus N. Uzonensis” chromosome. Contigs are separated by white space in all rings. Predicted coding sequences (rings 1+2), RNA genes (ring 3), regions of genomic plasticity compared to “Candidatus Nitrosopumilus maritimus” (ring 4) or “Candidatus Cenarchaeum symbiosum” (ring 5), and local nucleotide composition measures (rings 6+7) are shown. Very short features were enlarged to enhance visibility.

Fig. S2. Phylogenetic tree showing a selection of AmoA sequences obtained from hot environments that are closely related to the sequence of “Candidatus Nitrosotenuis uzonensis“. The tree shows only group I.1a-affiliated sequences, while group I.1b-related sequences have been used as outgroup. If known, temperature and pH of the sample from which the respective sequence had been obtained is indicated. The number in parentheses indicates the number of sequences within the group. The scale bar equals 3 % estimated sequence divergence.

Fig. S3. Dot plots representing genome-wide alignments of “Candidatus Nitrosotenuis uzonensis” to (a) “Candidatus Nitrosopumilus maritimus”, (b) “Candidatus Nitrosoarchaeum limnia”, and (c) “Candidatus Cenarchaeum symbiosum”. Forward matches are shown in red, while reverse matches are shown in blue.
References


Fig. 1
Fig. 2
Tab. 1. Overview of key features of the “Ca. Nitrosotenuis uzonensis” genome.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genome size</td>
<td>1,649,125 bp</td>
</tr>
<tr>
<td>Average GC-content</td>
<td>42.2 %</td>
</tr>
<tr>
<td>Number of scaffolds</td>
<td>1</td>
</tr>
<tr>
<td>Number of contigs</td>
<td>14</td>
</tr>
<tr>
<td>Number of genomic objects (CDS, fCDS, tRNAs, rRNAs)</td>
<td>1,999</td>
</tr>
<tr>
<td>Number of CDS</td>
<td>1,958</td>
</tr>
<tr>
<td>Number of CDS with predicted function</td>
<td>217 (11.1 %)</td>
</tr>
<tr>
<td>CDS density</td>
<td>1.19 CDS/kb</td>
</tr>
<tr>
<td>Average CDS length</td>
<td>766.3 bp</td>
</tr>
<tr>
<td>Average intergenic length</td>
<td>98.5 bp</td>
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<tr>
<td>Protein coding density</td>
<td>90.2 %</td>
</tr>
<tr>
<td>Number of 16S-23S rRNA operons</td>
<td>1</td>
</tr>
<tr>
<td>Number of 5S rRNAs</td>
<td>1</td>
</tr>
<tr>
<td>Number of tRNAs</td>
<td>38</td>
</tr>
<tr>
<td>Number of amoA, amoB, amoC genes</td>
<td>1 / 1 / 2</td>
</tr>
<tr>
<td>Number of accA/pccB and hcd genes</td>
<td>1 / 1</td>
</tr>
</tbody>
</table>

CDS, predicted coding sequences; fCDS, fragmented CDS
Ca. Nitrosotenuis uzonensis
1,649,125 bp
Fig. S2
Fig. S3
**Tab. S1.** Oligonucleotide probes used for FISH and/or CARD-FISH.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence (5'-3')</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arch915</td>
<td>GTG CTC CCC CGC CAA TTC CT</td>
<td><em>Archaea</em></td>
<td>(Stahl and Amann, 1991)</td>
</tr>
<tr>
<td>Cren512</td>
<td>CGG CGG CTG ACA CCA G</td>
<td>most <em>Crenarchaeota</em></td>
<td>(Jurgens et al., 2000)</td>
</tr>
<tr>
<td>EUB338</td>
<td>GCT GCC TCC CGT AGG AGT</td>
<td>most <em>Bacteria</em></td>
<td>(Amann et al., 1990)</td>
</tr>
<tr>
<td>EUB338 II</td>
<td>GCA GCC ACC CGT AGG TGT</td>
<td><em>Planctomycetes</em></td>
<td>(Daims et al., 1999)</td>
</tr>
<tr>
<td>EUB338 III</td>
<td>GCT GCC ACC CGT AGG TGT</td>
<td><em>Verrucomicrobiales</em></td>
<td>(Daims et al., 1999)</td>
</tr>
<tr>
<td>N4-1130</td>
<td>AGT GCC CAC TCA TCG CGT</td>
<td>novel betaproteobacterial species co-occurring in AOA enrichments from a Uzon caldera hot spring</td>
<td>-</td>
</tr>
<tr>
<td>N4Ggen-1009</td>
<td>CAC TCC CCC GTC TCC GGG</td>
<td>novel betaproteobacterial species co-occurring in AOA enrichments from Russian hot springs</td>
<td>-</td>
</tr>
<tr>
<td>NonEUB</td>
<td>ACT CCT ACG GGA GGC AGC</td>
<td>test for unspecific binding</td>
<td>(Wallner et al., 1993)</td>
</tr>
<tr>
<td>Nso190</td>
<td>CGA TCC CCT GCT TTT CTC C</td>
<td>betaproteobacterial ammonia-oxidizing bacteria</td>
<td>(Mobarry et al., 1996)</td>
</tr>
<tr>
<td>Nso1225</td>
<td>CGC CAT TGT ATT ACG TGT GA</td>
<td>betaproteobacterial ammonia-oxidizing bacteria</td>
<td>(Mobarry et al., 1996)</td>
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References


Table S2. Oligonucleotide primers used in PCR-amplifications.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Target gene</th>
<th>Specificity</th>
<th>Annealing temperature [°C]</th>
<th>Reference</th>
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<tbody>
<tr>
<td>21F</td>
<td>16S rRNA</td>
<td>Archaea</td>
<td>56</td>
<td>(DeLong, 1992)</td>
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<tr>
<td>616V</td>
<td>16S rRNA</td>
<td>Bacteria</td>
<td>54</td>
<td>(Juretschko et al., 1998)</td>
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<tr>
<td>1492R</td>
<td>16S rRNA</td>
<td>Bacteria and Archaea</td>
<td>54-56</td>
<td>(Lane, 1991)</td>
</tr>
<tr>
<td>Arch-amoAF</td>
<td>amoA</td>
<td>archaeal ammonia-oxidizers</td>
<td>54</td>
<td>(Francis et al., 2005)</td>
</tr>
<tr>
<td>Arch-amoAR</td>
<td>amoA</td>
<td>archaeal ammonia-oxidizers</td>
<td>54</td>
<td>(Francis et al., 2005)</td>
</tr>
<tr>
<td>amoA1F</td>
<td>amoA</td>
<td>bacterial ammonia-oxidizers</td>
<td>50</td>
<td>(Rotthauwe et al., 1997)</td>
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<tr>
<td>amoA2R</td>
<td>amoA</td>
<td>bacterial ammonia-oxidizers</td>
<td>50</td>
<td>(Rotthauwe et al., 1997)</td>
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<tr>
<td>CrenAmo2.1F</td>
<td>amoB</td>
<td>archaeal ammonia-oxidizers</td>
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<tr>
<td>CrenAmo2.1R</td>
<td>amoB</td>
<td>archaeal ammonia-oxidizers</td>
<td>54</td>
<td>(Könneke et al., 2005)</td>
</tr>
<tr>
<td>hcd911F</td>
<td>hcd</td>
<td>archaea</td>
<td>55</td>
<td>(Offre et al.)</td>
</tr>
<tr>
<td>hcd1267R</td>
<td>hcd</td>
<td>archaea</td>
<td>55</td>
<td>(Offre et al.)</td>
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References


Tab. S3. Enzymes of the carbon-fixing 3-hydroxypropionate/4-hydroxybutyrate pathway in “Candidatus Nitrosotenuis uzonensis”.

<table>
<thead>
<tr>
<th>N. uzonensis</th>
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<th>nt position</th>
<th>Annotation</th>
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<tr>
<td>NITUZv1_140005</td>
<td>1,316,825</td>
<td>1,318,021</td>
<td>Acetoacetyl-CoA-ketothiolase</td>
<td>Nmar_1631</td>
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<tr>
<td>NITUZv1_140127</td>
<td>1,422,146</td>
<td>1,424,242</td>
<td>4-Hydroxybutyryl-CoA synthetase/4-Hydroxybutyrate-CoA ligase</td>
<td>Nmar_0206</td>
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<tr>
<td>NITUZv1_140137</td>
<td>1,429,896</td>
<td>1,431,284</td>
<td>Succinyl-CoA reductase</td>
<td>Nmar_1608</td>
</tr>
<tr>
<td>NITUZv1_140140</td>
<td>1,436,697</td>
<td>1,438,196</td>
<td>4-Hydroxybutyryl-CoA dehydratase</td>
<td>Nmar_0207</td>
</tr>
<tr>
<td>NITUZv1_30440</td>
<td>440,730</td>
<td>441,125</td>
<td>Glyoxalase/bleomycin resistance protein/dioxygenase</td>
<td>Nmar_0953</td>
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<tr>
<td>NITUZv1_30441</td>
<td>441,126</td>
<td>442,718</td>
<td>Methylmalonyl-CoA epimerase, Methylmalonyl-CoAmutase</td>
<td>Nmar_0954</td>
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<tr>
<td>NITUZv1_30446</td>
<td>446,916</td>
<td>447,338</td>
<td>fragment of Methylmalonyl-CoA mutase large subunit (MCM-alpha)</td>
<td>Nmar_0958</td>
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<td>NITUZv1_30486</td>
<td>481,618</td>
<td>482,757</td>
<td>3-Hydroxybutyryl-CoA dehydrogenase</td>
<td>Nmar_1028</td>
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<tr>
<td>NITUZv1_40030</td>
<td>538,715</td>
<td>539,659</td>
<td>Succinate semialdehyde reductase (iron-containing alcohol dehydrogenase)</td>
<td>Nmar_1110, Nmar_0161</td>
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<td>NITUZv1_40192</td>
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<td>677,349</td>
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<td>Nmar_1308</td>
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<td>NITUZv1_40440</td>
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<td>Acetoacetyl-CoA-ketothiolase</td>
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<td>3-Hydroxypropionyl-CoA synthetase/Acryloyl-CoA reductase</td>
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<td>-</td>
<td>-</td>
<td>Malonyl-CoA reductase, Malonate semialdehyde reductase</td>
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</table>
Tab. S4. Genes implicated in the biochemistry for ammonia oxidation in “Candidatus Nitrosotenuis uzonensis”. The table is adapted from Walker et al., 2010.

<table>
<thead>
<tr>
<th><strong>N. uzonensis</strong></th>
<th><strong>nt position</strong></th>
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Reference

Tab. S5. List of genes implicated in chemotaxis that are present in the genome of “Candidatus Nitrosotenuis uzonensis”, but are absent in other sequenced AOA. -, absent; +, present

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Chapter V

Thaumarchaeotes abundant in refinery nitrifying sludges express amoA but do not live primarily from autotrophic ammonia oxidation

Under review in *Proc Natl Acad Sci U S A*
Thaumarchaeotes abundant in refinery nitrifying sludges express amoA but do not live primarily from autotrophic ammonia oxidation

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Abstract

Nitrification is a core process in the global nitrogen cycle that is essential for the functioning of many ecosystems. The discovery of autotrophic ammonia oxidizing archaea (AOA) within the phylum Thaumarchaeota has changed our perception of the microbiology of nitrification, in particular since their numerical dominance over ammonia oxidizing bacteria (AOB) in many environments has been revealed. These and other data have led to a widely held assumption that all _amoA_-carrying members of the Thaumarchaeota are autotrophic nitrifiers. In this study, 52 municipal and industrial wastewater treatment plants were screened for the presence of putative thaumarchaeotal AOA and AOB. Thaumarchaeota carrying _amoA_ were detected in high abundance only in four industrial plants. In one plant, thaumarchaeotes closely related to soil group I.1b Thaumarchaeota outnumbered AOB up to 10,000-fold, and their numbers were two to three orders of magnitude higher than could be sustained by autotrophic ammonia oxidation. Consistently, $^{14}$CO$_2$ fixation could not be detected in these archaea by fluorescence in situ hybridization (FISH) combined with microautoradiography, despite active nitrification in the activated sludge. Furthermore, _in situ_ transcription of archaeal _amoA_, and very weak _in situ_ labelling of crenarchaeol after addition of $^{13}$CO$_2$ was independent of the addition of ammonium. These data demonstrate that the energy metabolism of some _amoA_-carrying group I.1b Thaumarchaeota is not restricted to autotrophic nitrification and some are mixotrophs or heterotrophs.
**Introduction**

In recent years there have been a number of startling new discoveries in the biogeochemistry of the nitrogen cycle (1-3). Not least of these has been the demonstration that a novel group of archaea, now known to belong to the novel phylum Thaumarchaeota (4, 5) are capable of autotrophic ammonia oxidation (2). This physiology has in the meantime been confirmed for different lineages within this phylum (6-10). The widespread distribution and abundance of these AOA, has been shown through metagenomic surveys, targeted retrieval of archaeal 16S rRNA- and ammonia monooxygenase genes and analysis of characteristic archaeal lipids (11-13). Moreover, a prominent role for AOA relative to AOB in nitrification in soil, marine and geothermal systems has been revealed on the basis of quantitative gene abundance and expression data in relation to ammonia amendment and measured nitrification as well as by stable isotope probing (14-16). This has led to the widely held assumption that all amoA-carrying members of the Thaumarchaeota are capable of autotrophic nitrification, although a few reports suggested that some of these organisms might also be able to assimilate organic compounds like amino acids (17-19). Furthermore, PCR-based studies indicated that certain thaumarchaeotes might not carry amoA genes (19, 20) but these findings might be explained by primer mismatches to certain amoA sequences (21).

Ammonia oxidation as the rate-limiting step of nitrification is also a vital process in engineered biological systems such as wastewater treatment plants (WWTPs). The microbiology of nitrogen removal in WWTPs has been intensively studied and the application of molecular tools has led to the identification of the most abundant bacterial nitrifying populations in these systems. The population structure and dynamics of AOB in WWTPs has received particular attention (for a review see 22). By contrast there have been very few studies of putative AOA (pAOA) - the term we use in this study for amoA-carrying thaumarchaeotes - in engineered biological treatment systems (23-25). When quantitative analyses of AOB and pAOA have been conducted in some nitrifying WWTPs, the abundance of AOB has been shown to exceed that of pAOA by 2 to 3 orders of magnitude. In these cases pAOA were rarely detected at an abundance greater than $10^3$ ml$^{-1}$ (24, 26, 27). This is consistent with the failure to detect thaumarchaeotal sequences in an analysis of archaeal 16S rRNA genes in activated sludge (28). By contrast, higher abundances of pAOA were described in some Asian WTPs and a correlation between pAOA abundance and the ammonia concentration in the wastewater was postulated (29, 30). The significance of putative ammonia-oxidizing thaumarchaeota for nitrogen removal in WWTPs therefore remains unclear.
Here we report a survey of the diversity, abundance and activity of thaumarchaeotes in 52 municipal and industrial WWTPs in Europe and demonstrate that pAOA are not widespread in nitrifying reactors. pAOA were however abundant in a small number of industrial WWTPs. In one of these plants pAOA were 2 to 4 orders of magnitude more abundant than AOB but the measured numbers were far too great to be explained by the amount of ammonia removal occurring in this system. In this treatment plant AOB assimilated significantly higher amounts of bicarbonate under nitrifying conditions than pAOA. This, combined with the results from in situ labelling experiments with $^{13}$C-inorganic carbon and compound-specific carbon isotope data for archaeal lipids, showed that the pAOA in this plant employ an alternative mode of energy metabolism to autotrophic ammonia oxidation.

**Results**

**Detection of Thaumarchaeota in WWTPs.** In total, 35 domestic and 17 industrial WWTPs from different geographic locations were screened for the occurrence of pAOA. The WWTPs encompassed a wide range of reactor configurations and influent sewage compositions. 46 of the WWTPs exhibited high nitrification performance leading to the removal of at least 90% of the ammonia in the influent (Dataset S1 and (31)). Archaeal amoA genes (193 clones) affiliated with group I.1a and group I.1b Thaumarchaeota, were detected only in six industrial and one municipal WWTP (Fig. S1). From four of these WWTPs (refinery plants A, D, E and a pilot scale reactor from a tannery wastewater treatment plant, plant F) thaumarchaeotal 16S rRNA genes (102 clones) belonging to groups I.1a or I.1b could be retrieved using an archaea-specific PCR assay (Fig. 1). Consistent with this was the observation of thaumarchaeote-specific signals with 16S rRNA catalyzed reporter deposition-fluorescence in situ hybridisation (CARD-FISH) in only these four industrial WWTPs, using previously published as well as a newly designed probes (Fig. 2; Table 1; Table S1). The specificity of the CARD-FISH assay was confirmed by double-hybridizations of thaumarchaeotal subgroup-specific probes with the more general probes Cren512 and Arch915. Group I.1b-affiliated Thaumarchaeota in the sludges of plants D and E were detected using a probe specific to cloned 16S rRNA sequences recovered from the WWTPs (Thaum1162). Thaum1162 exclusively hybridized to large, coccoid cells with an average cell diameter of 1-2 µm that mainly occurred in aggregates consisting of up to 200 cells. In contrast, pAOA detected in plants F and A which hybridized with the group I.1a specific probe Cren537 were smaller and formed irregular-shaped colonies with a diameter of 5-8 µm that consisted of less
than 70 cells (Fig. 2). With one exception (brewery WWTP Rapp-Kutzenhausen), AOB were
detected by FISH with specific probes in all nitrifying sludges analyzed.
According to the molecular survey data, sludge from the nitrifying refinery plant D contained
a single thaumarchaeotal operational taxonomic unit affiliated with group I.1b, which
occurred at a relatively high abundance. Due to the low diversity of group I.1b
Thaumarchaeota and the co-occurrence of AOB in plant D, the pAOA of this plant were
selected for subsequent in-depth analyses to assess their role in nitrification.

Quantification of putative AOA and AOB in refinery plant D. 16S rRNA gene- as well as
archaeal amoA gene-based quantitative PCR data from two parallel reactors of the nitrifying
refinery plant D confirmed the high abundance of pAOA in this system. From both reactors
four activated sludge samples obtained at different times were analyzed by qPCR and the
numbers of pAOA (assuming a single 16S rRNA and amoA gene per thaumarchaeotal
genome (32)) ranged from 6.76 to 8.42 log\(_{10}\) cells ml\(^{-1}\). The ratios of thaumarchaeotal 16S
rRNA genes to thaumarchaeotal amoA genes in the eight samples varied between 0.8 and 2.2
(Table 1). In accordance with the qPCR data, quantitative CARD-FISH analysis of two
samples from one of the reactors of refinery plant D demonstrated that between 7.95 and 8.04
log\(_{10}\) group I.1b thaumarchaeotal cells ml\(^{-1}\) hybridized with probe Thaum1162 (Table 1),
representing up to 5% of the total cell counts in this system. The four samples from both
reactors of refinery plant D were also analyzed by qPCR targeting the amoA gene of
betaproteobacterial AOB and using FISH targeting AOB 16S rRNA. Bacterial amoA genes
were detected in consistent numbers in all samples with an average of 2 x 10\(^4\) AOB cells ml\(^{-1}\)
(4.3 log\(_{10}\) copies ml\(^{-1}\) sludge; Table 1). AOB abundance determined by FISH was somewhat
higher than the abundance determined by qPCR (5.6 to 6.4 log\(_{10}\) copies ml\(^{-1}\) sludge, Table 1).
Consistent with the detection of Thaumarchaeota at high abundance in refinery plant D using
nucleic acid based approaches, high amounts of crenarchaeol (GDGT-I, Fig. S2), a
characteristic glycerol dibiphytanyl glycerol tetraether (GDGT) of thaumarchaeotes was
detected (33-35) (Table 2). Reactors A and B of this plant contained 8.8 and 10.9 µg
crenarchaeol g\(^{-1}\) dry sludge respectively, while a control reactor (Ingolstadt plant), in which
no Thaumarchaeota were detected by nucleic acid-based methods, contained only 0.1 µg
crenarchaeol g\(^{-1}\) dry sludge. Furthermore both reactors of plant D contained 0.4 µg g\(^{-1}\) dry
sludge of the crenarchaeol regioisomer GDGT-VI (Table 2), which is relatively abundant in
the group I.1b AOA Nitrososphaera gargensis (35), while this compound was below the
detection limit in the control plant (Table 2). Assuming that all GDGTs are derived from
living cells, then it is possible to estimate the number of pAOA cells in the sludge based on the lipid concentrations. If we further assume that all pAOA cells are spheres with a diameter of 1.5 µm as is indicated by FISH data, and that 1 µm$^2$ of archaeal cell membrane contains ca. $1.7 \times 10^5$ GDGT molecules (36), then the pAOA would contain 2.6 femtograms of GDGT per cell. Using the summed concentrations of thaumarchaeotai GDGTs (Table 2) and a sludge water content of 99% (based on MLSS (mixed liquor suspended solids) measurements from the reactors), we estimate cell numbers in the order of $6 \times 10^7$ cells ml$^{-1}$ ($7.8 \log_{10}$ cells/ml) which is very similar to our qPCR and FISH based measurements (Table 1).

**Modelling the abundance of ammonia-oxidizing microorganisms in refinery plant D.** To determine whether the abundance of pAOA in refinery plant D could be explained by autotrophic ammonia oxidation alone, we used the nitrification model developed by Rittman (37, 38), to estimate ammonia oxidizer biomass in relation to ammonia removal in wastewater treatment reactors. To do this we conservatively assumed that 20% of the ammonia was consumed by assimilation and estimated the growth yield of pAOA based on the data presented by Könneke et al. (2). The calculated growth yield was 1.2 g dw/mol N which compared favorably with the range of growth yields reported for AOB in the literature (0.1 to 1.4 g dw/mol N; (39)). This is not surprising given the known thermodynamic constraints on growth yields (40, 41). We therefore used the same growth yield and other physiological parameters for pAOA that have previously been used to estimate the abundance of AOB in WWTPs based on ammonia removal (37, 38, 42). The possibility that there was an additional contribution to reduced nitrogen in the system from nitrogen fixation was precluded by measurement of nitrogen fixation in sludge samples from refinery plant D by isotope ratio mass spectrometry, which proved negative (Table S2).

The modeling of data from samples from the two reactors in refinery plant D taken on four separate occasions indicated that the ammonia removal in the reactor would support a population of autotrophic ammonia oxidizers of between 4.48 to 5.00 $\log_{10}$ pAOA cells ml$^{-1}$, which is about two to three orders of magnitude lower than the population sizes estimated from quantitative FISH, or qPCR of archaeal amoA or 16S rRNA genes (Table 1). Predicted pAOA numbers are only marginally higher when calculations are based on total Kjeldahl nitrogen (TKN) removal. However, the numbers of autotrophic ammonia oxidizing bacteria estimated from the model (5.36 to 5.88 $\log_{10}$ AOB cells ml$^{-1}$; note that modeled AOB numbers are higher than AOA numbers due to the smaller size of AOB cells in the reactors) are in line with, though higher than, the abundance of AOB amoA genes measured in the
same samples (4.15 to 4.45 log_{10} AOB cells ml^{-1}) and fit well with AOB numbers measured by FISH (5.59 to 6.35 log_{10} AOB cells ml^{-1}; Table 1). Quantitation of AOB by qPCR may underestimate the true numbers of AOB present in activated sludge since DNA extraction from AOB microcolonies is known to be difficult (43) and thus cell specific ammonia oxidation rates (CSAOR) were estimated based on the FISH data. Inferred CSAORs of the AOB in the sludge, were comparable to values reported for reference strains which range from 0.9 to 53 fmol/cell/h (44) and CSAORs estimated \textit{in situ} (0.03 to 43 fmol/cell/h; (42, 45), Table 1). CSAORs based on the abundance of pAOA were typically much lower (Table 1). Collectively, these data strongly call in to doubt the notion that the \textit{amoA}-carrying Thaumarchaeota in this system gain most of their energy from chemoautotrophic ammonia oxidation.

**Metabolic activity and ecophysiology of putative AOA in refinery plant D.** To test whether the sludge from refinery plant D retained its nitrifying capacity during lab incubation experiments, live sludge was amended with 0.5 mM NH$_4$Cl and the ammonium concentration was followed for 5 h. In a parallel experiment, 1µg ml$^{-1}$ of diphtheria toxin, an inhibitor of translation in eukarya and archaia (46), was added to the sludge. In both experiments, more than 90% of the added ammonium was removed within 2.5 hours (Fig. S3). Furthermore, transcription of \textit{amoA} genes from group I.1b Thaumarchaeota was detected in incubated sludge from refinery plant D both with and without addition of 2 mM NH$_4$Cl as demonstrated by RT-PCR (Fig. S4). Cloning and sequencing of the \textit{amoA} RT-PCR product revealed sequences identical to the \textit{amoA} sequence cluster obtained from genomic DNA of plant D during the survey experiments (Fig. S1; Ref D_11). Subsequently, we combined FISH with microautoradiography (47) to analyze whether the pAOA and AOB in plant D incorporated $^{14}$C-inorganic carbon in the presence of ammonium. Ammonium chloride (0.5 mM) was added to sludge from plant D (07.05.2008, reactor A) and FISH with microautoradiography was conducted after six hours of incubation. Unexpectedly, the pAOA showed no incorporation of radioactive bicarbonate, while this was strongly assimilated by the AOB (Fig. 3). Absence of autotrophic activity detectable by microautoradiography of the pAOA in the presence of ammonia was reproduced in an independent control experiment (09.10.2008, reactor A). In addition, only AOB were shown to fix labelled inorganic carbon in samples from refinery E which, like refinery D had consistently higher abundance of pAOA than AOB (Fig. 3C to F).
Determination of the $\delta^{13}C$ value of the biphytanates released by ether cleavage from GDGTs in the sludge of reactor B of plant D revealed that the crenarchaeol-derived biphytanates (C40:2 and C40:3; Fig. S2) were depleted by ca. 17 ‰ compared to the dissolved inorganic carbon (mean $\delta^{13}C$ of -27.7 ‰) of the sludge supernatant (Table S3). Labelling experiments using crenarchaeol as a biomarker for Thaumarchaeota, were performed with nitrifying biomass from refinery plant D (reactor A, 9.10.2009). The sludge was incubated with 99 atom% $^{13}C$-bicarbonate for 18 hours with or without addition of 1 mM NH$_4$Cl. After incubation the crenarchaeol-derived biphytanates C40:2 and C40:3 were very weakly $^{13}C$-labelled ($\Delta \delta^{13}C$ of ca. 7 ‰; Table S3), independent of the addition of ammonium.

In some archaea including the autotrophic AOA *Nitrosopumilus maritimus*, inorganic carbon fixation is achieved through the 3-hydroxypropionate/4-hydroxybutyrate cycle (32, 48). A key enzyme in this pathway is 4-hydroxybutyryl-CoA dehydratase encoded by the *hcd* gene. Using a recently published PCR assay (10), we were unable to detect *hcd* genes in the sludge of plant D.

**Discussion**

Thaumarchaeota have been shown to play a significant role in nitrification in marine and terrestrial environments (14, 15, 49-51). Despite these findings it is till unclear whether all *amoA*-carrying thaumarchaeotes detected in these environments indeed live from autotrophic ammonia oxidation and their potential role for nitrogen removal in engineered biological systems has not been clarified yet (23-25, 30). We report an extensive survey of the distribution, abundance and activity of pAOA in diverse WWTPs. The only occasional occurrence of Thaumarchaeota among 52 plants suggests that they are generally minor contributors to nitrification of most wastewaters. Out of 46 nitrifying sludges only four industrial WWTPs harboured Thaumarchaeota at relative abundances that were above the detection limit of CARD-FISH (Fig. 2). Three of these four sludges originate from petroleum refinery WWTPs, which may reflect a certain habitat preference of the detected pAOA. The consistently high abundance of a single Thaumarchaeota OTU throughout the study period in refinery plant D demonstrates that they did not originate from allochthonous inflow (i.e. from surrounding terrestrial habitats), but rather accounted for a substantial part of the indigenous microbial community in this plant. Here, both CARD-FISH cell counts and abundance of *amoA* and 16S rRNA genes repeatedly revealed thaumarchaeotal cell abundance of $10^7$-$10^8$ cells ml$^{-1}$. The quantification of crenarchaeol complemented our nucleic acid-based
approaches and strongly supported the high abundance of Thaumarchaeota detected by FISH and qPCR in this sludge. In all other sludges tested, in which pAOA were not detected by CARD-FISH, betaproteobacterial AOB prevailed (with one exception - Rapp-Kutzenhausen) and were therefore most likely responsible for ammonia oxidation in these plants. In addition, AOB numbers estimated on the basis of a nitrification model were consistent with AOB numbers measured by FISH in the refinery D WWTP, even though they were present at 3 to 4 orders of magnitude lower abundance than the pAOA. Taken together, these data strongly support the contention that betaproteobacterial ammonia oxidizers are the main agents driving the first step of nitrification in most WWTPs (52).

Our initial assumption was that substantially higher numbers of pAOA in refinery WWTP plant D must implicate them as the main agents of ammonia-oxidation in these systems. However, when we modelled the expected abundance of AOA based on ammonia or TKN removal in the plant, the amount of reduced nitrogen oxidized was only sufficient to support a population of autotrophically-growing ammonia-oxidizing microbes of ca. 0.1 to 1 % of that observed for the Thaumarchaeota in the system (Table 1). In turn, the predicted numbers were in line with the measured abundance of AOB (Table 1) suggesting that the AOB could very well be the solely responsible agents for nitrification. Moreover CSAORs calculated based on pAOA abundance were either vanishingly small or at the lower end of CSAORs estimates from other systems (Table 1; there is only a single report of a CSAOR lower than 0.22 fmol/cell/h, (44)). AOB based CSAORs were by contrast directly in line with what has been measured in pure cultures and the majority of WWTP (42, 44). Of course, the model of ammonia oxidation includes important assumptions regarding the growth yield and endogenous biomass decay terms. However, to obtain predicted numbers, which approach those measured would require a 10- to 100- fold increase in the yield and a corresponding decrease in endogenous biomass decay. Thus, even if the values used are not completely accurate it is unlikely that the model is incorrect by the 2 to 4 orders of magnitude that the replicated qPCR, CARD-FISH and archaeal lipid data suggest. One of the most influential factors affecting the modelled cell numbers is the cell biovolume term, where clearly, smaller cells would lead to higher cell numbers in the calculations. We addressed this by determining pAOA biovolume empirically from CARD-FISH images. This method has been further verified by tests with cultured AOA to ensure that the applied CARD-FISH procedure does not lead to significant changes in cell diameter. The predicted numbers would be consistent with the experimentally determined cell counts only if we assume a diameter of the AOA
cells in plant D of ca. 0.1 µm (compared to the measured 1.4 µm, in line with cell sizes of closely related thaumarchaeotes (53, 54). The primary conclusion of the modelling analysis is that the abundance of the Thaumarchaeota, determined independently using CARD-FISH, qPCR and lipid analysis, is far too great to be explained by chemolithoautotrophic ammonia oxidation alone. Consistent with a non-autotrophic lifestyle, the pAOA in the sludge of plant D did not incorporate $^{14}$C-bicarbonate in the presence of ammonia at levels detectable by microautoradiography. By contrast, in the same experiment, strong $^{14}$C-labelling was observed for the AOB, which were present in this sludge at much lower abundance than the thaumarchaeotes (Fig. 3). $^{13}$C-bicarbonate incorporation into thaumarchaeotal lipids measured by highly sensitive isotope ratio mass spectrometry was also very limited, and could conceivably have occurred as a result of heterotrophic CO$_2$ fixation (55), and was independent of ammonia addition (Table S3). Furthermore, we were unable to detect hcd genes in the sludge of plant D by using a recently published PCR assay (10). As hcd genes encode 4-hydroxybutyryl-CoA dehydratase, a key component of the 3-hydroxypropionate/4-hydroxybutyrate cycle for autotrophic bicarbonate fixation in the genomes of all analyzed AOA (8, 32, 48, 56), the failure of this PCR assay would be expected, if the Thaumarchaeota in refinery plant D are not autotrophic. Likewise, ammonia oxidation in sludge from refinery plant D was not inhibited by diphtheria toxin, an inhibitor of archaeal protein synthesis (Fig. S3; (46)) and although expression of archaeal amoA genes was detected in refinery plant D samples this too was independent of added ammonia (Fig. S4).

A role for Thaumarchaeota in nitrification in the refinery D WWTP was further discounted following failure and subsequent recovery of nitrification in the plant. Following a period of more than five years where the pAOA were stably present in the plant (Table 1 and Dataset S1), pAOA were no longer detectable by CARD-FISH. The loss of the thaumarchaeotal population coincided with an incident at the refinery, which required the deployment of fire fighting foam which entered the refinery WWTP the day prior to a sampling expedition. This induced a period of poor performance and when we returned to sample the plant following recovery of the treatment plant performance, despite complete re-establishment of nitrification in the system, no Thaumarchaeota could be detected in the sludge. This unambiguously confirmed our conclusion that they are not essential for ammonia oxidation in this plant.

Taken together, these data strongly suggest that the AOB present in the refinery D WWTP are responsible for the ammonia oxidation in this system and that the amoA-carrying
Thaumarchaeota are in fact not chemolithoautotrophic ammonia oxidizers. If that is the case, what then is their role in this WWTP where they account for a considerable part of the microbial population? One possibility is that these Thaumarchaeota, which represented a single OTU within the group I.1b, are indeed ammonia oxidizers, but can gain additional energy and carbon from other substrates. This, however, is inconsistent with the fact that the levels of ammonia oxidation observed, are compatible with the population size of AOB which were demonstrated to be active in chemolithoautotrophic ammonia oxidation in the reactor (Table 1 and Fig. 3). The \( \text{amoA} \)-carrying group I.1b Thaumarchaeota might also be heterotrophs using some unknown organic compounds present in the wastewater as a carbon and energy source (18, 19).

Taking this into account one piece of data remains apparently inconsistent with a heterotrophic lifestyle for the thaumarchaeotes from the refinery WWTP. Stable carbon isotope analysis of crenarchaeol demonstrated that this was isotopically depleted by about 17 \(^{\text{‰}}\) relative to inorganic carbon (Table S3). This pattern is consistent with autotrophic carbon fixation and is observed in marine systems, where the group I.1a Thaumarchaeota are considered to be autotrophic (57, 58). However typically lipids are \( ^{13}\text{C} \) depleted relative to the carbon source used by between 5 and 20 \(^{\text{‰}}\) (59). DIC in the refinery treatment plant has a \( \delta^{13}\text{C} \) of -27.7 \(^{\text{‰}}\) and \( \delta^{13}\text{C} \) sludge biomass was -28.4 ± 0.6 \(^{\text{‰}}\) (Table S2). This is consistent with the main sources of organic carbon in the refinery wastewater being products of crude oil processing, as crude oil \( \delta^{13}\text{C} \) can range from -24 to -35 \(^{\text{‰}}\) depending on oil source, maturity and migration (60). Thus archaeal lipids with a \( \delta^{13}\text{C} \) of -46.2 ± 1.9\(^{\text{‰}}\) would not be inconsistent with heterotrophic growth of \( \text{amoA} \)-carrying group I.1b Thaumarchaeota, on organic by-products of the crude oil refining process.

This being the case what is the potential role of the ammonia monooxygenase (AMO) homologue carried by non-autotrophic group I.1b Thaumarchaeota? Bacterial AMO display a high substrate flexibility and are well known for co-metabolising hydrocarbons (61), which is also reflected in the evolutionary relatedness of bacterial ammonia- and methane-monooxygenases (62). This is in line with the recent discovery of a novel monooxygenase from the AMO and pMMO family that catalyzes butane oxidation (63). As bacterial and archaeal ammonia monooxygenases are phylogenetically highly divergent, it is plausible that within this diversity of archaeal ammonia monooxygenases some may in fact use substrates other than ammonia (64). Thus, we hypothesize that the Thaumarchaeota in the petroleum refinery plant D could in fact utilize hydrocarbons or other compounds present in wastewater from crude oil refining processes that are activated by monooxygenases. To test this we
conducted a FISH-MAR survey using radiolabelled amino acids, pyruvate, acetate, benzoate, and phenol, none of which were assimilated by the thaumarchaeotes in the sludge. However, as refinery activated sludge is exposed to a vast complexity of compounds the lack of incorporation of the tested compounds does not exclude a heterotrophic life mode of these thaumarchaeotes.

Given the known relationship between monooxygenase involved in methane and ammonia oxidation one further possibility is that the Thaumarchaeota might be methane oxidizers. Indeed we have detected \textit{mcrA} sequences (Fig. S5) and lipids (Table 2) from methanogens in the sludge and shown that it has potential for biogenic methane generation at low rates (78 ± 1.2 nmoles/ml/day) suggesting that methane might be a carbon source for the Thaumarchaeota we detected. However, the $\delta^{13}$C of the thaumarchaeal lipids was not sufficiently $^{13}$C-depleted to support this suggestion.

In summary, our data show that not all \textit{amoA}-carrying Thaumarchaeota are ammonia oxidizers and several lines of circumstantial evidence point towards them being heterotrophs, which use organic carbon compounds present in the refinery wastewater. Thus, results from metagenomic, metatranscriptomic or cloning studies based on the detection of putative archaenal ammonia monooxygenase gene sequences or their transcripts in environmental samples have to be interpreted with caution, because the retrieved DNA or RNA fragments do not necessarily originate from ammonia oxidizers. In this context it is particularly interesting to note that the group I.1b Thaumarchaeota of refinery plant D are closely related (based on 16S rRNA and \textit{amoA}) to sequences found in various soils (Fig. S1 and Fig. 1). Thus, the role of AOA in carbon sequestration in terrestrial systems might be less prominent than recently suggested (9). As generally group I.1b dominates the Thaumarchaeota communities in soil (65), a metabolism other than ammonia oxidation of some members of this group has important implications for our understanding of the contribution of these organisms to N- and C-cycling in terrestrial ecosystems.

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sludges. We thank C. Schleper, M. Stieglmeier, M. Schmid, and M. Könneke for helpful discussions.

Material and Methods

**WWTPs sampling.** Samples from 52 WWTPs (Dataset S1) were collected and used for DNA extraction or fixed with paraformaldehyde for FISH.

**Detailed methods are provided in SI Materials and Methods.**

**Nucleotide accession numbers.** Sequences of 16S rRNA and *amoA* gene fragments determined in this study have been deposited at GenBank with the following accession numbers: HQ316962-HQ316983 (16S rRNA), and HQ316984 -HQ317060 (*amoA*).
### References


31. Pickering RL (2008) How hard is the biomass working?: Can cell specific uptake rates be used to optimise the performance of bacterial biomass in wastewater treatment plants? PhD thesis (Newcastle University, Newcastle upon Tyne, UK).


Chapter V


Fig. 1. Phylogeny of the 102 thaumarchaeotal 16S rRNA sequences recovered from four activated sludge samples (plant abbreviations are identical as in Figure 1). Based on different treeing methods a consensus tree was constructed. Sequences were grouped based on a sequence identity cut-off >99%. Open squares, bootstrap support >70%, closed square, bootstrap support >90% using 100 iterations (Maximum Parsimony). Open diamonds, bootstrap support >70%, closed diamonds, bootstrap support >90% using 1000 iterations (Neighbour Joining). Scale bar represents 10% estimated sequence divergence. Brackets indicate specificities of probes Cren537 and Thaum1162. Clones from refinery plant D are labelled in red.
Fig. 2. Detection of Thaumarchaeota in industrial WWTPs by CARD-FISH. (A) Plant F sludge hybridized with probe Cren512 targeting most Cren- and Thaumarchaeota (B) the same cells hybridized by group I.1a-specific probe Cren537. (C) Refinery plant D sludge hybridized with the general archaeal probe Arch915. (D) the same cells targeted by the clone-specific probe Thaum1162. (E) Refinery plant D sludge hybridized with probe Arch915. Note the density of AOA colonies reflecting their high relative abundance. (F) Refinery plant E sludge hybridized with probe Thaum1162 after sonication. Scale bar indicates 5 µm.
Fig. 3. FISH-microautoradiographic analyses of AOB and pAOA in refinery WWTPs under nitrifying conditions in the presence of $^{14}$CO$_2$. Panels A, C and E display FISH signals obtained with probe Arch915 (panel A, red signals) and a probe mix (probes Nso190, Nso1225, Neu, NmV and Nso192) targeting betaproteobacterial AOB (panel C and E, green signals). Lower panels B, D and F shows the corresponding microautoradiography images. Panels A to D show samples from refinery D (07.05.2008) and Panels E and F show samples from Refinery E (26.03.2008). AOB but not pAOA showed autotrophic carbon fixation during this experiment. Bar corresponds to 5 µm.
**Table 1.** Measured and modeled abundance of AOA and AOB in different samples from the parallel reactors A and B of plant D as determined by qPCR (amoA and 16S rRNA gene), FISH and modeling. Cell specific ammonia oxidation rates (CSAOR) calculated using the quantitative AOB FISH data are also presented. A mix of probes Nso190 and Nso1225 labelled with the same fluorophore was used for detection of AOB by FISH. For quantification of AOA by CARD-FISH, probes Cren1162 and Cren512 were applied.

### Putative ammonia-oxidizing archaea (pAOA)

<table>
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<tr>
<th>Refinery D</th>
<th>log archaeal amoA gene copies/ml</th>
<th>log cluster I.1b 16S rRNA gene copies/ml</th>
<th>Ratio amoA/16S rRNA gene copies</th>
<th>Modeled log pAOA abundance&lt;sup&gt;1&lt;/sup&gt;</th>
<th>pAOA CSAOR (fmol/cell/h)&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Modeled log FISH counts cells/ml</th>
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<td>7.71±0.07</td>
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<td>7.10±0.30</td>
<td>6.85±0.16</td>
<td>6.99±0.22</td>
<td>0.8</td>
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### Ammonia-oxidizing bacteria (AOB)

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<th>Modeled log AOB abundance&lt;sup&gt;2&lt;/sup&gt;</th>
<th>AOB CSAOR (fmol/cell/h)&lt;sup&gt;3&lt;/sup&gt;</th>
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Table 2. Concentrations of glycerol dibiphytanyl glycerol tetraethers (GDGTs and glycerol trialkyl glycerol tetraethers (GTGT) from refinery plant D (7.5.2008) and the Ingolstadt WWTP, which served as a control plant without putative AOA detectable by molecular methods.

<table>
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<tr>
<th></th>
<th>GTGT -I</th>
<th>GDGT -II</th>
<th>GDGT -III</th>
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<th>GDGT -V</th>
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<td>Reactor B</td>
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<td>(control)</td>
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<td>92.9</td>
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Supplementary material and methods

**WWTPs analyzed.** In total 52 WWTPs were analyzed in this study. Details regarding these plants including the type of the wastewater and key operational parameters are summarized in Dataset S1. This dataset also provides information on the sampling dates.

**Sampling for FISH and DNA extraction.** The oil refineries WWTPs (plants A-E) and the pilot scale reactor from a tannery waste plant (plant F) from the UK were sampled as follows: triplicate biofilm and mixed liquor grab samples, respectively, were collected and samples for both DNA extraction and FISH were initially preserved in ethanol (final concentration 50% (v/v)). Samples were kept at 4°C during transportation to the lab and then stored at -20°C. For FISH, subsamples were taken from the ethanol preserved samples and additionally fixed using paraformaldehyde (PFA). For this purpose, samples were centrifuged and the pellet was washed once with phosphate buffered saline (PBS) (10 mM potassium phosphate, 150 mM sodium chloride, pH 7.2) and resuspended in PBS before adding 3 volumes of a PFA solution (3% final concentration, in PBS). Samples from Germany, Switzerland and Austria (“continental European”) were immediately fixed for FISH by adding 1 to 3 volumes of a PFA solution (in PBS, final concentration of 2% or 3%, respectively). All these samples were incubated at 4°C and further processed within 24 h after sampling. Subsequently, all WWTP samples were washed in PBS to remove residual PFA. After a final centrifugation step the pellets were resuspended in PBS: ethanol (1:1) and stored at -20°C. For DNA analysis samples from continental European WWTPs were collected in 50 ml plastic vials without fixative, cooled and shipped to the lab for further processing and storage at -20°C.

**DNA extraction.** For the UK sludges, 200 µl of ethanol fixed biofilm sample (refinery plant A) or 250 µl of activated sludge samples (refineries B-C, E sampling S1-2 and refinery plant D, sampling S1-4) were centrifuged and the pellets were resuspended in 250 µl of double distilled H₂O to normalize the differences in mixed liquor suspended solids (MLSS) content in the different sludges. DNA was extracted directly from these 250 µl samples. All manipulations were taken into account when calculating gene abundances from qPCR data. For DNA extraction of sludges investigated by Pickering (2008; (11)) 250 µl of mixed liquor was used. Before extraction, samples were physically disrupted by bead-beating with a Ribolyser (Hybaid Ltd, Middlesex, UK) for 30 seconds at a speed of 6.5 m/s. All DNA extracts were recovered using a BIO 101 FastDNA Spin Kit for Soil (Q-Biogene, UK),
following the manufacturer’s instructions. DNA of the continental European sludges were extracted from pelleted sludge or biofilm using the Power Soil DNA Isolation Kit, (MO BIO Laboratories, Inc) according to the manufacturer’s protocol.

**PCR screening for the presence of putative AOA, mcrA genes and hcd genes.** Out of the 52 sampled WWTPs, 49 were surveyed for the presence of the thaumarchaeotal amoA gene by PCR using the primers Arch-amoAF and Arch-amoAR (2). The three WWTPs from Vienna (HKA, OMV, VUW) were only screened by CARD-FISH for the presence of thaumarchaeotes. Samples from the UK were screened using the following PCR conditions: 95°C for 5 min; 30 cycles consisting of 94°C for 45 s, 53°C for 60 s, and 72°C for 60 s; and 72°C for 15 min. The continental European WWTP samples were screened according to the following PCR protocol 94°C for 5 min, 35 cycles of 94°C (40 s), 56°C (40 s), 72°C (20 s) and a final extension at 72°C (5 min). The recovered PCR products were cloned using the TOPO TA cloning kit for sequencing (Invitrogen™ Ltd, Paisley, UK).

The archaeal 16S rRNA gene was amplified from DNA extracted from refinery plants A-E with previously published primers 20f (3) and Uni1392 (4) using the following PCR conditions: 95°C for 3 min; 30 cycles consisting of 94°C for 30 s, 56°C for 30 s, and 72°C for 110 s. Final elongation was performed at 72°C for 7 min and then at 60°C for 5 min. The recovered PCR products were cloned using the TOPO TA cloning kit for sequencing (Invitrogen™ Ltd, Paisley, UK). mcrA genes, encoding methyl coenzyme M reductase of methanogenic archaea, were amplified using primers mcrA-MLf and mcrA-MLr (5) at an annealing temperature of 55°C. 30 PCR-cycles were used for amplification of target DNA. Cloning and sequencing was performed as described by (6). Amplification of hcd genes from AOA encoding 4-hydroxybutyryl-CoA dehydratase in Plant D sludge DNA was conducted as follows. DNA was extracted and purified via a phenol/chloroform-extraction including a bead-beating step (30 sec, 6 m/s; adapted from (7)). PCR using primers hcd911F and hcd1267R (8) was conducted at a range of annealing temperatures from 52-55°C. These assays all gave negative results with DNA extracted from refinery plant D, while positive controls consistently yielded a specific PCR product.

**Phylogenetic analyses and probe design.** For phylogenetic analyses of the 16S rRNA and AmoA sequences the ARB program package (9) was used. Based on the ARB-SILVA 16S rRNA database SSURef_92_tree_silva_opt.arb (10) maximum parsimony (100 bootstrap-resamplings), distance-matrix (ARB Neighbour-Joining, Jukes Cantor correction with 1000
bootstrap-resamplings) and maximum-likelihood methods with a 50% conservation filter for archaeal sequences were applied for inferring 16S rRNA trees. Partial sequences were added to the consensus tree using the parsimony criterion without altering the overall topology. For phylogenetic analysis of the ammonia monooxygenase subunit A (AmoA) 188 amino acid positions were considered for maximum Parsimony (100 bootstrap-resamplings), distance-matrix (ARB Neighbour-Joining with the JTT correction factor) and maximum-likelihood calculations (Phylip-ML). The reference database used for these analyses contained 3689 archaeal and bacterial sequences. From the different 16S rRNA and AmoA trees strict consensus trees were constructed for each marker molecule. Furthermore, based on the recovered 16S rRNA sequences of the group I.1b Thaumarchaeota from plant D and plant E sludges, the new oligonucleotide probe Thaum1162 was designed using the PROBE_DESIGN tool of the ARB program package (9) and used for in situ detection of the respective Thaumarchaeota.

**Quantitative PCR.** Quantification of amoA and 16S rRNA genes was conducted on DNA from three independent biological replicate samples from the reactors. Q-PCR assays were performed in 96-well plates using a thermocycler IQ™5 (Bio-Rad, Hercules, USA) according to the manufacture’s instructions. All primers and probes used for quantitative PCR fully matched the recovered thaumarchaeotal amoA and archaeal 16S rRNA genes from plant D. The abundance of 16S rRNA genes from group I.1b Thaumarchaeota in plant D was quantified as described by (11) using 10 pM of primers 771F (5’-ACGGTGAGGGATGAAAGCT-3’) and 957R (5’-CGGCGTTGACTCTACATTG-3’) and the following PCR conditions: initial denaturation at 95°C for 7 min, followed by 55 cycles of denaturation at 95°C for 30 s, primer annealing at 54°C for 45 s, and extension at 72 ºC for 45 s. The archaeal amoA gene was quantified as described by (12) using 20 pM each of primers amo196F (5’-GGWGTKCCRGGRACWGCMAC-3’), amo277R (5’-CRATGAAGTCRTAHGGRTA DCC-3’), and 10 pM of the TaqMan probe (5’-FAM-CAAACCAWGCWCCYT TKGCDACCCA-TAMRA-3’, Thermo Electron GmbH, Ulm, Germany) under the following PCR conditions: initial denaturation at 95°C for 7 min, followed by 40 cycles of denaturation at 95°C for 15 s, primer annealing at 55 ºC for 40 s, extension at 72 ºC for 20 s. The bacterial amoA gene was quantified as described by (13) using 6 pM of primers AmoA 1-F (5’-GGGGTTTCTACTGTATTGTG-3’), and AmoA 2-R (5’-CCCTCGSAAAGCCTTCTTTC-3’) and the following PCR conditions: initial denaturation at 95°C for 7 min, followed by 40 cycles of denaturation at 95°C for 20 s, primer annealing at
For calibration of the 16S rRNA gene and amoA gene assays standards were generated from cloned sequences recovered from plant D by amplification from plasmids using vector based primers. DNA concentrations of standards were determined using a DNA spectrophotometer NanoDrop® ND-1000 (Wilmington, USA). Standards were serially diluted to concentrations ranging from $10^8$ to $10^3$ copies µl$^{-1}$. Standards were run in duplicate and the environmental samples were run in triplicate. Besides primers each PCR reaction (20 µl) contained 3 µl DNA template and either a Taqman-probe (see above) or 1% SYBR® Green I (Sigma, Poole, UK; 10000 x conc. in DMSO) in 10 µl iQ-Supermix PCR reagent (BIO-RAD, Hercules, CA, USA) and 6 µl of molecular biology grade water. Two negative controls without template were included in each assay as well as two samples spiked with standard DNA to check for PCR inhibition. After SYBR Green assays melting curves of sample- and clone-derived PCR products were compared to confirm the presence of only a single peak to demonstrate the specificity of the PCR reaction. After calibration with the standards the abundance of amoA and 16S rRNA genes per ml sludge was calculated. Values of PCR efficiency/slope/R$^2$ of the standard curve were (69%/-4.395/0.988) for the 16S rRNA gene of Thaumarchaeota group I.1b, (100%/-3.317/0.958) for AOB amoA, and (83%/-3.593/0.992) for AOA amoA.

FISH and CARD-FISH. For quantification of total cell numbers and AOA numbers, PFA-fixed samples of plant D were sonicated for 30 s on ice (Bandelin, Sonopuls, cycle 2, amplitude 20%, Germany) and were subsequently filtered on polycarbonate membranes (0.2 µm pore size, 47 mm, Millipore, Germany). For quantification of AOA, polycarbonate filters were sectioned directly after filtration. Untreated sections were used for counts of the total cell number (see below), whereas the remaining sections were used for CARD-FISH according to (14) with slight modifications. Specifically, filter membranes were mounted with 0.1% agarose. Endogenous peroxidases were inactivated by a treatment with 0.15% H$_2$O$_2$ in methanol for 30 min. Afterwards membranes were washed in water and ethanol (1 min each). Cells were permeabilized by proteinase K (15 µg/ml, Sigma, in 0.1 M Tris, 0.01 EDTA, pH 8.0, 5-8 minutes at room temperature) with subsequent washing in water (1 min) and inactivation of proteinase K by 0.01 M HCl for 20 min. Buffers used in hybridization, washing and amplification were as described previously (15). Samples and peroxidase-labelled probes were hybridized for 3 h at 46 °C. Probe Thaum1162 was hybridized for 16-20 h to ensure hybridization in 16S rRNA regions of decreased accessibility (16). The signal was amplified using carboxy-fluorescein-labelled tyramides for 45-60 min at 46°C. Subsequently,
samples were washed first in water and then in ethanol (1 min each). Finally, DNA was stained by DAPI (1 µg/ml). AOA cell numbers were determined by calculating the ratio of the total cell number on untreated filter sections (thereby avoiding a potential bias due to lysis of some microbial cells due to the application of the CARD-FISH protocol) and AOA number on proteinase K treated filter sections. Standard FISH for AOB and Archaea using Cy3- and FITC-labelled probes was performed on PFA-fixed samples according to (17). AOB probes Nso1225, NEU, NmV, Nso192 cluster 6a and the respective competitors were mixed in equimolar concentrations and hybridized at 35 % formamide (AOB mix).

**Modelling autotrophic ammonia oxidizer abundance.** The abundance of ammonia oxidizers in the plant D was estimated from levels of ammonia removal using the model developed by Rittman (18, 19). Firstly ammonia oxidizer biomass \( X_{AO} \) was estimated using the equation.

\[
X_{AO} = \frac{\theta x}{\theta} \left[ \frac{Y_{AO}}{1 + b_{AO} * \theta x} * \Delta Ammonia \right]
\]

Where \( X_{AO} \) is the biomass of ammonia oxidizers in mg/l, \( \theta \) is the mean cell residence time in days, \( \theta \) is the hydraulic retention time in days, \( Y_{AO} \) is the growth yield of ammonia oxidizers (0.34 kg VSS / kg NH\(_4^+\)-N), \( b_{AO} \) is the endogenous respiration constant of ammonia oxidizers (0.15 d\(^{-1}\)) and \( \Delta Ammonia \) is the difference in influent and effluent ammonia concentrations in mg/l.

The yield value used was decided based on calculation of the growth yield of calculated growth yield of *N. maritimus* based on the data presented in (20). The yield was 1.15 g dw/mol N which compared favorably with the range of growth yields reported for AOB in the literature (0.1 to 1.4 g dw/mol N; 21). We therefore use the same growth yield and other physiological parameters that have previously been used to estimate the abundance of AOB in WWTP based on ammonia removal (18, 19). Hydraulic parameters such as mean cell residence time and hydraulic retention time and ammonia removal data required by the model were calculated from operational data provided by the plant operators. The AOA biomass values obtained from the model were converted to biovolume using a conversion factor of 310 fg C/µm\(^3\) (21). Cell numbers were calculated from biovolume data on the basis of the mean cell size of thaumarchaeotes (d=1.47 ± 0.24 µm diameter, n=50 cells, Fig. 2) and the AOB (d=0.75 ± 0.08 µm diameter, n=25 cells, Fig. 2) using CARD-FISH confocal laser scanning microscopy from sludge samples from refinery plant D.
**Archaea amoA mRNA analysis.** Sludge from plant D (S5) was aliquoted (4 ml in 50 ml tubes) in three replicates and 2.0 mM NH₄Cl was added. In addition, three replicates without NH₄Cl amendment served as control treatments. The sludge was incubated overnight at 30°C without shaking. Subsequently, the sludge samples were stored at -80°C until RNA extraction. RNA was extracted using TRIzol (Invitrogen, USA) according to the manufacturer’s instructions and finally dissolved in 100 µl water. 5 µl community RNA served as template for reverse transcription and subsequent PCR amplification using the Super Script III One Step RT-PCR kit (Promega, USA) according to the manufacturer’s protocol. To check for possible DNA contamination parallel reactions were set up without the initial reverse transcription. For reverse transcription and PCR amplification primers Arch-amoAF and Arch-amoAR (2) were used with the following conditions: reverse transcription was performed at 45°C for 45 min and was followed by enzyme inactivation of 2 min at 94°C. Subsequent PCR was conducted as follows: denaturation at 94°C for 30 sec, primer annealing at 56°C for 1 min, elongation at 68°C for 2 min and a final elongation at 68°C for 7 min. The PCR product was cloned and sequenced as described above.

**Combined FISH and microautoradiography.** Activated sludge from plant D, reactor A was sampled on 7.5.2008 (S5), chilled and transported to the laboratory within 48 h. In addition, activated sludge from this plant (reactor A) was sampled at 9.10.2008 (S6), transported to the lab without chilling (to test whether chilling negatively affects the metabolic activity of the members of the Thaumarchaeota) and was processed within 30 h. For sampling S5 the sludge was diluted ten-fold with filtered (0.2 µm pore size) supernatant and was preincubated for 3 h each at 26°C with 0.05 mM NH₄Cl. The preincubated sludge was subsequently aliquoted into 10 ml vials with a total activated sludge volume of 2 ml. These were amended (duplicate incubations) with 0.15, 0.5 and 5 mM NH₄Cl, respectively. After addition of 7.5 µCi [¹⁴C]-bicarbonate (Hanke Laboratory Products, Germany) the samples were incubated for 6h at 26°C (*in situ* T) without shaking. For sampling S6 the sludge was diluted four-fold and directly amended with 2 µCi [¹⁴C]-bicarbonate and 0.5 mM of ammonium and incubated for 4h at 31°C (*in situ* T) without shaking. In both experiments controls were included by using sludges without addition of ammonium as well as with sludges treated with 1.8% formaldehyde prior to the incubation in order to check for physiological activity of the thaumarchaeotes without added substrate and for chemography, respectively. After incubation, biomass was fixed with 1.8% formaldehyde as
described by (17). FISH staining was performed as described above. MAR was performed as described earlier with modifications (22). The hybridized samples were dipped in preheated (48°C) LM-1 emulsion (Amersham, USA), exposed for 7-28 days at 4°C in the dark and developed in Kodak D19 (40 g liter/l of distilled water) before microscopy. For sampling S6 the FISH-MAR procedure was altered by using slightly different chemicals and by application of membrane filters according to the protocol of Alonso et al. (23).

Diphtheria toxin inhibition experiment and ammonium measurements. 3 ml of undiluted plant D sludge (S5, 7.5.2008) was amended with 0.5 mM of NH₄Cl and with diphtheria toxin (1 µg/ml, Sigma, Germany) and was incubated in 10 ml vials in triplicates for 5 h at 27°C without shaking. Controls were performed without amendment of diphtheria toxin. Finally, the remaining ammonium concentration was measured in the supernatant according to (24). Nitrate formation was tested semi-quantitatively by nitrate test strips (Merck, Germany).

Nitrogen fixation assay. Cooled (4 °) and un-cooled sludge samples were sent and stored at 4 °C and room temperature (~25 °C), respectively, before analyses were performed. Nitrogen fixation was assessed by following the incorporation of the stable isotope ¹⁵N of ¹⁵N₂ into the microbial biomass as described elsewhere (25). Briefly, 3 ml sludge samples were incubated at 27 °C in an artificial ¹⁵N₂:O₂ atmosphere (80:20%, v:v; ¹⁵N₂ at 98 at % ¹⁵N, Cambridge Isotope Laboratories, MA, USA) in headspace vials (18 ml, butyl rubber septa) for 14.5 h under constant horizontal shaking (100 rpm). Untreated controls were incubated under the same conditions but with ambient N₂ to determine the natural abundance of ¹⁵N in the sludge. After incubation, sludge samples were centrifuged at 12,000 g, the supernatant decanted and the pelleted biomass dried at 60 °C overnight. Samples were finely ground in a ball mill (MM2000; Retsch GmbH & Co. KG, Haan, Germany) and aliquots of 0.5 mg dry material were weighed in tin capsules. The abundances of ¹⁵N (at % ¹⁵N) were determined with a continuous-flow isotope ratio mass spectrometer (Delta Advantage; Thermo, Germany), linked to an elemental analyzer. All experiments were done in triplicate.

Measurements of crenarchaeol concentrations in two activated sludges.
For lipid analysis 2 x 50 ml of refinery D sludge (S5, 7.5.2008) reactor A, 2 x 50 ml of refinery D, reactor B sludge and and 1 x 50 ml from Ingolstadt sludge (May 2008) were centrifuged and lyophilized. Pre-weighed, freeze-dried reactor material was ultrasonically extracted three times with an organic solvent mixture of dichloromethane (DCM):methanol
(MeOH) (2:1, v/v). Total lipid extracts were collected in a round bottom flask, evaporated to dryness under rotary vacuum, re-dissolved in DCM and dried again over Na$_2$SO$_4$. To each extract, 0.1 µg of a C$_{46}$ internal standard was added before it was chromatographed over activated Al$_2$O$_3$. The GDGT-containing fraction was eluted with DCM:MeOH (1:1, v/v), collected and dried under a stream of N$_2$, re-dissolved in hexane:isopropanol (99:1, v/v) and filtered through a 0.45µm pore size, 4 mm diameter, Teflon filter.

Archaeal GDGTs were analyzed using high performance liquid chromatography (HPLC) – atmospheric pressure chemical ionization mass spectrometry (APCI-MS) by applying conditions slightly modified as previously reported (26, 27). Analyses were performed using an HP 1100 series LC/MSD (Palo-Alto, CA, US) equipped with an auto-injector and Chemstation chromatography manager software. For the first 5 min elution was isocratic with 99% hexane and 1% isopropanol, followed by a gradient to 1.8% isopropanol in 45 min. The flow rate was 0.2 ml/min. Separation was achieved on a Prevail Cyano column (2.1 x 150 mm, 3 µm; Alltech, Deerfield, IL, USA) maintained at 30°C. After each analysis the column was cleaned by back flushing hexane/propanol (9:1, v:v) at 0.2 ml/min for 10 min. Detection was achieved by positive ion APCI with the following conditions: nebulizer pressure (N$_2$) 60 psi, vaporizer temperature 400°C, drying gas (N$_2$) flow 6 l/min and temperature 200°C, corona current 5 µA, capillary voltage -3kV. Archaeal GDGTs were detected with single ion monitoring of their protonated molecules [M + H]$^+$]. SIM parameters were set to detect protonated molecules of common isoprenoid-tetraethers (m/z 1304, 1302, 1300, 1298, 1296, 1294, 1292, 1290, and 1288) as well as the internal standard (m/z 744), with a dwell time of 237 ms per ion. Archaeal tetraethers were quantified according to (28). Due to co-elution with crenarchaeol, concentrations of GDGT-4 (m/z 1294) corrected for the [M+H+2]$^+$ isotope peak of crenarchaeol (29).

**Compound-specific $^{13}$C analysis of GDGT-derived biphytanes.** Activated sludge from plant D (reactor A) was sampled at 9.10.2009 (S6), transported to the lab without chilling and was processed within 30 h. The sludge was amended with 0.5 mM [13C]-labelled sodium bicarbonate (99% atom $^{13}$C, Sigma, Germany) and 1.0 mM of NH$_4$Cl and aliquoted into triplicate flasks (50 ml) with a total sludge volume of 15 ml. Sludge incubated without addition of ammonia served as control. As an additional control, sludge amended with ammonia and bicarbonate was inhibited by mercury chloride to reveal unspecific bicarbonate adsorption. The sludge was then incubated in the dark for 18 h at 31°C without shaking. Nitrate formation was tested semi-quantitatively by nitrate test strips. After centrifugation the
solid sludge fraction was processed further and GDGTs were extracted. GDGTs were subjected to ether bond cleavage as described by (30) and analyzed by gas chromatography coupled to mass spectrometry (GC/MS) for biphytanes on a ThermoFinnigan TRACE gas chromatograph coupled with a ThermoFinnigan DSQ quadrupole mass spectrometer. Compound-specific $\delta^{13}C$ analyses were performed on the aliphatic fraction using an Agilent 6800 GC coupled to a ThermoFisher Delta V isotope ratio monitoring mass spectrometer (IRMS). Isotope values were measured against calibrated external reference gas. The $\delta^{13}C$ values for individual compounds are reported in the standard delta notation against the Vienna Pee Dee Belenite (VPDB) standard.
References


## Dataset S1

<table>
<thead>
<tr>
<th>No.</th>
<th>WWTP</th>
<th>Sampling date</th>
<th>origin of wastewater</th>
<th>reactor type</th>
<th>T (°C)</th>
<th>pH</th>
<th>Conductivity (µS/cm)</th>
<th>NH₄⁺ (mM)</th>
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<td>DO (mg/l)</td>
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<td>TKN (mg/l) (influent/effluent)</td>
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n.d. = not determined

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COD, chemical oxygen demand  
BOD, biological oxygen demand  
DO, dissolved oxygen  
MVLSS, Mixed liquor volatile suspended solids  
SRT, sludge retention time  
TKN, Total Kjeldahl nitrogen  
DIC, differential internal cycling  
SBR, sequencing batch reactor

*a* non-nitrifying  
*b* final membrane filtration  
*c* sampling period 30.03.2005- 21.06.2006 (8x)  
*d* averaged values  
*e* MLSS (Mixed liquor suspended solids  
*f* total N (no TKN available)  
*g* or not available
|------|------|------|------|------|-------|------|------|--------|
Fig. S1. Phylogeny of the 193 thaumarchaeotal AmoA sequences recovered from seven activated sludge samples. Based on different treeing methods a consensus tree was constructed considering 188 amino acid positions. Sequences were grouped based on a nucleotide sequence identity cut-off >99%. Open squares indicate bootstrap support >70%, closed square shows a bootstrap support >90% using 100 iterations (Maximum Parsimony). Cluster designations were made according to Park et al. 2006 (Appl. Environ. Microbiol. 72:5643-5647). Clones were retrieved from six industrial WWTPs [animal rendering and dairy plant Ampfing (AM), animal rendering plant Lyss (TBA); oil refinery plants A, D, E and tannery plant F (Ref A, D, E, and TanF)] and the municipal plant Altmannstein (AL). Clones from refinery plant D are labelled in red. Scale bar represents 10% estimated sequence divergence.
Fig. S2. Structures of archaeal GTGT and GDGTs referred to in Table 2.
Fig. S3. Ammonium concentration in plant D sludge (07.05.2008) amended with 0.5 mM ammonium at the beginning of the experiment ($T_0$), and after 2.5h of incubation at 27°C in the absence (−DT) or presence of 1 μg/ml diphtheria toxin (+DT). After 2.5 h in both samples nitrate formation could be detected with test strips.
Fig. S4. Detection of mRNA of thaumarchaeotal amoA in ammonia-amended (+) and unamended (-) plant D sludge (07.05.2008). Left side: mRNA detection by RT-PCR. Middle lanes: PCR control for DNA contamination in the same samples. Each experiment was performed with three replicates. On the right side of the gel an amoA PCR negative and positive control are shown, respectively. M, size marker. It should be noted that amoA expression was detected in two of the three replicates independent of the addition of ammonium.
Fig. S5. Phylogenetic analysis of McrA sequences (152 amino acid positions considered) inferred from cloned mcrA gene fragments from plant D. Based on different treeing methods a consensus tree was constructed using a strict consensus rule. Two short sequences from the plant D sludge were added to the tree without changing the overall tree topology. Open squares, bootstrap support >70%; closed square, bootstrap support >90%; using 100 iterations (Maximum Parsimony). Scale bar represents 10% estimated sequence divergence.
Table S1. Oligonucleotide probes used for FISH or CARD-FISH.

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<td>Most Bacteria</td>
<td>GCT GCC TCC CGT AGG AGT</td>
<td>HRP</td>
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<td>Amann et al., (1990)</td>
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<tr>
<td>NONsense</td>
<td>Control probe</td>
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<td>HRP</td>
<td>10-35%</td>
<td>Hatzenpichler et al. (2008)</td>
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<tr>
<td>Arch915</td>
<td>Most Archaea</td>
<td>GTG CTC CCC CGC CAA TCC</td>
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<td>Cren512</td>
<td>Thaumarchaeota, most crenarchaeota except Thermoprotei</td>
<td>CGG CGG CTG ACA CCA G</td>
<td>HRP</td>
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</tr>
<tr>
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<td>CGG CGG CTG GCA CCA G</td>
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<td>CGG CGG CTG GCA CCC G</td>
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<td></td>
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<tr>
<td>Cren537</td>
<td>Marine group I.1a thaumarchaeota</td>
<td>TGA CCA CTT GAG GTG CTG</td>
<td>HRP</td>
<td>20%</td>
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</tr>
<tr>
<td>Thaum1162</td>
<td>Subcluster of the thaumarchaeotia cluster I.1b</td>
<td>TTC TTC CTG CTG AGC GAC</td>
<td>HRP</td>
<td>20%</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>competitor for Thaum1162</td>
<td>TTC TTC CTG CTG AGC GGC</td>
<td>HRP</td>
<td>20%</td>
<td>This study</td>
</tr>
<tr>
<td>Eury499</td>
<td>most Euryarchaeota</td>
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<td>HRP</td>
<td>20%</td>
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<tr>
<td>NEU</td>
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<td>CCC TCT TGC TGC ACT CTA</td>
<td>Cy3</td>
<td>35%</td>
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</tr>
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<td>competitor for NEU</td>
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<td>HRP</td>
<td>20%</td>
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<td>Nso192 cluster 6a</td>
<td>N. oligotropha lineage (cluster 6a)</td>
<td>CTT TCG ATC CCC TAC TTT CC</td>
<td>Cy3</td>
<td>35%</td>
<td>Adamczyk et al., (2003)</td>
</tr>
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<td>CTT TCG ATC CCC TGC TCC</td>
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<td>35%</td>
<td>Adamczyk et al., (2003)</td>
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<tr>
<td>Nnv (Nomob)</td>
<td>Nitrosococcus mobilis</td>
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<td>Cy3</td>
<td>35%</td>
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<tr>
<td>Nso1225</td>
<td>most betaproteobacterial ammonia oxidizing bacteria</td>
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<td>Cy3</td>
<td>35%</td>
<td>Mobarry et al. (1996)</td>
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<tr>
<td>Nso190</td>
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<td>CGA TCC CCT GCT TTT CTC C</td>
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<td>40%</td>
<td>Mobarry et al. (1996)</td>
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References:
**Table S2.** Incorporation of $^{15}$N from $^{15}$N$_2$ into biomass in refinery D sludge and $\delta^{13}$C of sludge organic matter.

### October 2010

<table>
<thead>
<tr>
<th>Identifier</th>
<th>at% C</th>
<th>$\delta^{13}$C/$^{12}$C</th>
<th>at% N</th>
<th>$\delta^{15}$N/$^{14}$N</th>
<th>$\delta^{15}$N/$^{14}$N mean</th>
<th>Std-dev</th>
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### November 2010

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<th>at% N</th>
<th>$\delta^{15}$N/$^{14}$N</th>
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4°C, RT – sludge samples were sent and stored at 4 °C and room temperature (~25 °C), respectively
All incubations were conducted at 27°C
Std-dev – standard deviation
at% - atom percent
I, II, III - replicate number
Table S3. $\delta^{13}$C analysis of GDGT-derived biphytanes from 13-C labelling experiments. Incorporation of $^{13}$C into crenarchaeol–derived biphytanes in refinery plant D (sampling S6, 9.10.2009) amended with 0.5 mM $^{13}$C-labelled bicarbonate with or without addition of 1 mM NH$_4^+$. Errors indicate the standard deviation from 3-4 measurements.

<table>
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<th>C40:0</th>
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<th>C40:2</th>
<th>C40:3$^c$</th>
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<tr>
<td>$T_0$</td>
<td>-56.0 ±1.7</td>
<td>-43.1 ±0.7</td>
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<td>-46.2 ±1.9</td>
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<td>$T_{(\text{end})}^a$</td>
<td>-48.1 ±1.0</td>
<td>-37.8 ±1.1</td>
<td>-38.2 ±0.5</td>
<td>-38.9 ±0.8</td>
<td>-52.8 ±0.3</td>
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<td>$T_{(\text{end})}^b$</td>
<td>-41.7 ±0.6</td>
<td>-35.5 ±1.5</td>
<td>-37.4 ±0.6</td>
<td>-39.5 ±1.1</td>
<td>nd</td>
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</table>

$^a$experiment with addition of 13-C labelled bicarbonate
$^b$experiment with addition of 13-C labelled bicarbonate and NH$_4^+$
$^c$C40:0, C40:1, C40:2 and C40:3 refer to biphytanes with 0 to 3 cycloalkyl rings, respectively (Fig S3). C40:3 biphytane contains two cyclopentane and a cyclohexane ring, which is thought to be exclusively derived from crenarchaeol and thus to be highly specific to thaumarchaeotes.
Chapter VI

Identification and preliminary ecophysiologica
and genomic characterization of novel
betaproteobacteria co-enriched with
ammonia-oxidizing archaea

Study description
Identification and preliminary ecophysiological and genomic characterization of novel betaproteobacteria co-enriched with ammonia-oxidizing archaea

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Abstract

Despite the crucial importance of microbes catalyzing the aerobic oxidation of ammonia to nitrite in the environment our knowledge about their diversity and ecophysiology is still very limited. The recent discovery of ammonia-oxidizing archaea (AOA) has changed our perception of the key-players in nitrification in many habitats and revealed our lack of knowledge about the metabolic capacities of a microbial group ubiquitous in nature. These unexpected new insights raise the question whether also yet undiscovered lineages of AOB exist. Here we report on a novel clade affiliated to the beta-subclass of *Proteobacteria* not closely related to known AOB that thrive in significant numbers in two ammonia-oxidizing enrichments recently obtained from two geographically distinct Russian geothermal springs. While one of these cultures constitutes a co-enrichment of a member of this new clade with the AOA “*Candidatus Nitrososphaera gargensis*”, in the second culture no known AOA or AOB could be detected. Incubations with labelled bicarbonate in the presence of ammonia as sole source of energy demonstrated the potential for autotrophic growth of the novel microbes. However, all key genes of ammonia oxidation of known AOB are missing from the yet incomplete genome of one of the enriched microbes. Hence, no solid conclusion concerning the organisms’ physiology can be presented at this point. This manuscript summarizes the data obtained over the past five years on the identification, physiological characterization and preliminary genomic analysis of these novel bacteria and presents hypotheses regarding their metabolism. It will hopefully serve as a useful resource for planning future experiments to characterize these mysterious organisms.
Introduction

More than a century ago the first isolation of ammonia-oxidizing bacteria (AOB) by Sergei Winogradsky provided evidence for the existence of chemolithoautotrophic organisms (Winogradsky, 1890, 1892). His experiments revised the view of nitrification as a one-step biological process by demonstrating that AOB first aerobically oxidize ammonia to nitrite, which is then further oxidized to nitrate by nitrite-oxidizing bacteria (Winogradsky, 1890, 1892). Nitrification is now recognized as a central part of the biogeochemical nitrogen cycle and has been postulated to exist since the first oxygenation of Earth’s oceans (Garvin et al., 2009; Godfrey and Falkowski, 2009; Canfield et al., 2010; Vlaeminck et al., 2011). Our understanding of the diversity and biochemistry of AOB has been hampered by the fact that AOB are notoriously recalcitrant to cultivation attempts and grow only slowly. Today, only 16 AOB-species representing five distinct bacterial genera within the beta- and gamma-subclasses of Proteobacteria are known (Koops et al., 2003; Bock and Wagner, 2006). Three of these genera were already described by Winogradsky (Winogradsky, 1892; Winogradsky and Winogradsky, 1933) and the last discovery of a new AOB genus, *Nitrosovibrio*, was published more than three decades ago (Harms et al., 1976).

The successful isolation of an ammonia-oxidizing archaeon (AOA) (Köneke et al., 2005) and the subsequent discovery that AOA outnumber their bacterial counterparts in many habitats (e.g. (Leininger et al., 2006; Wuchter et al., 2006; He et al., 2007; Lam et al., 2007; Beman et al., 2008; Schauss et al., 2009)) fundamentally revised our view of nitrogen cycling in the environment (Prosser and Nicol, 2008; Erguder et al., 2009; Schleper and Nicol, 2011). Surprisingly, information from all yet available AOA genomes suggests that their biochemistry for ammonia oxidation is fundamentally different from that of AOB (Hallam et al., 2006; Walker et al., 2010; Schleper and Nicol, 2011). Both groups rely on the ammonia monooxygenase (Amo), a member of a diverse family of copper-containing membrane-associated enzymes, for the oxidation of ammonia. However, the reaction-product probably differs in these two groups of organisms (Walker et al., 2010; Schleper and Nicol, 2011; Klotz, unpublished), because AOA lack important downstream factors such as the hydroxylamine oxidoreductase (Hao), cytochrome c or other iron-containing proteins (Walker et al., 2010; Schleper and Nicol, 2011) known from AOB (Arp et al., 2007; Klotz and Stein, 2008). Besides AOA and AOB, the autotrophic methane oxidizer *Methylococcus capsulatus* strain Bath (Ward et al., 2004) as well as some heterotrophic microbes (Moir et al., 1996; Crossman et al., 1997; Stouthamer et al., 1997) engage in Amo-based nitrification.
The novel insights into the metabolic distinctiveness of AOA are mirrored by the recognition of the phylogenetic uniqueness of these microbes. Recent comparative genomic analyses revealed, that members of this functional guild form a novel phylum within the archaeal domain, termed *Thaumarchaeota* (Brochier-Armanet et al., 2008; Gupta and Shami, 2010; Spang et al., 2010).

Here we report on a new (genus-level) clade affiliated with the beta-subclass of *Proteobacteria* (in the following referred to as betaproteobacteria) that are not closely affiliated with any ammonia oxidizer, but were highly enriched in two ammonia-oxidizing enrichment cultures we recently obtained from two geographically distant Russian geothermal springs. While in one of these enrichments the ammonia-oxidizing thaumarchaeote *Candidatus Nitrososphaera gargensis* is also present (Hatzenpichler et al., 2008), in the second culture no known AOA or AOB could be detected. We present results that indicate that the enriched novel betaproteobacteria may constitute a yet overlooked group of ammonia-oxidizers. However, all key genes required for ammonia oxidation by known AOB are absent in the yet incomplete genome of one of these organisms and it is therefore not possible at this stage to make solid conclusions regarding their actual physiology. This manuscript presents a summary of the experiments performed for identification, ecophysiological characterization and genomic analysis of these novel betaproteobacteria during the last five years and discusses hypotheses regarding their metabolism.
Materials and methods

Enrichment cultures

Two ammonia-oxidizing enrichment cultures inoculated with samples from terrestrial thermal springs located in the Buryatia Republic (Garga spring) (Lebedeva et al., 2005; Hatzenpichler et al., 2008) and in the Uzon caldera on the Kamchatka peninsula, respectively, were maintained for nine (Buryatia) and four (Kamchatka) years (for an overview of all experiments please refer to Fig. 1). In the first phase of these experiments, the influence of different incubation temperatures on ammonia oxidation was evaluated. For the Buryatian and Kamchatkan enrichment a temperature range of 37-60 °C and 37-55 °C was tested, respectively. The enrichment medium contained 54.4 mg KH$_2$PO$_4$, 74.4 mg KCl, 49.3 mg MgSO$_4$ 7H$_2$O, 584 mg NaCl, 33.8 µg MnSO$_4$, 49.4 µg H$_3$BO$_3$, 43.1 µg ZnSO$_4$ 7H$_2$O, 37.1 µg (NH$_4$)$_6$Mo$_7$O$_{24}$ 4H$_2$O, 97.3 mg FeSO$_4$, 5H$_2$O, 25.0 µg CuSO$_4$ 2H$_2$O L$^{-1}$. Furthermore, either 147 mg CaCl$_2$ or 4.0 g CaCO$_3$ L$^{-1}$ (partial precipitation was observed) was added. Within the last two years only CaCO$_3$-based medium was used. The cultures were grown aerobically in the dark without shaking in 100 mL or 300 mL Erlenmeyer flasks closed with aluminium caps or 100 mL flasks closed with provided plastic caps (Schott) in medium containing 1-5 mM ammonium (NH$_4$Cl) and ~5 mM sodium bicarbonate (NaHCO$_3$) at a starting pH of 7.4-7.8. In the growing cultures, pH was adjusted using 1 % bicarbonate solution or 0.1 M sodium hydroxide (NaOH). During the entire enrichment period, fresh medium was periodically inoculated 1:10 with parent enrichment culture to avoid inhibiting accumulation of nitrite. The concentrations of ammonium, nitrite and nitrate were measured regularly photometrically (Hatzenpichler et al., 2008; Bollmann et al., 2011), by using Merckoquant test stripes for nitrite and nitrate (Merck) or via HPLC-analysis as recently described (Hatzenpichler et al., 2008).

In the early stages of enrichment, the pH indicator cresol-red (Sigma) was added to the medium to allow for visual inspection of medium (colour change from violet to yellow at pH <7.2). In later stages (2007 and 2008 for the Buryatian and Kamchatkan culture, respectively), cresol-red was avoided, and the pH was regularly checked via an inoLab pH level 1 pH-meter (Inolab). When the pH dropped below 7.0, it was readjusted to starting pH using either sodium hydroxide or sodium bicarbonate solution (see above).
Amplification, cloning, sequencing and phylogenetic analyses of 16S rRNA, amo, and cbbL genes from the enrichment cultures

PCR amplifications of 16S rRNA and amo genes were performed on multiple occasions (Fig. 1) during the enrichment from DNA obtained via either phenol/chloroform-extraction including a bead-beating step (30 sec, 6 m s⁻¹; adapted from (Lueders et al., 2004)), or via the Power Soil kit (MOBio Laboratories) following the instructions of the manufacturer, including a 10 min vortexing step. PCR reaction mixtures contained 2 mM MgCl₂ and 1 U of Taq-polymerase (Fermentas Life Sciences, St. Leon-Rot, Germany) in combination with 50 pmol of the respective oligonucleotide primers. Unless stated otherwise in all reactions bovine serum albumin (BSA, 5 ng µl⁻¹ final concentration) was added, 35 cycles were used for amplification of target DNA and a final elongation step of 10 min at 72 °C was performed. For details on used primers please refer to Tab. S1.

Bacterial 16S rRNA genes were PCR-amplified using primers 616V and 630R (Juretschko et al., 1998) or 1492R (Lane, 1991) at an annealing temperature of 54 °C. In addition, bacterial 16S rRNA genes from the Buryatian culture were amplified using primers Nbur447 and Nbur1148 at an annealing temperature of 61 °C. After cloning (see below) the amplificate from this experiment, four clones were randomly picked and fully sequenced. Furthermore, a PCR using primers 616V, 630R and the oligonucleotide Nbur1148lock was performed at 61 °C. Nbur1148lock is identical in sequence to primer Nbur1148 but lacks the 3’OH-group, resulting in the inhibition of DNA-polymerization. After cloning the amplificate from this PCR experiment, 20 clones were randomly picked and sequenced. Archaeal amoA and amoB genes were amplified using primers Arch-amoAF and Arch-amoAR (Francis et al., 2005) and CrenAmo2.1F and CrenAmo2.1R (Könneke et al., 2005), respectively. For both primer pairs an annealing temperature of 54 °C was used. Amplification of bacterial amoA genes was unsuccessfully tried multiple times using primers amoA1F and amoA2R (Rotthauwe et al., 1997) and A189F and A682R (Holmes et al., 1995), respectively. Different cell lysis (including direct cell lysis through heating to 95 °C for 15 min prior to PCR) and DNA-extraction protocols (see above), DNA-polymerases (standard Taq-polymerase [Fermentas], Takara DNA-polymerase [Takara], HotStar DNA-polymerase [Quiagen]), primer stocks, MgCl₂-concentrations, annealing temperatures and cycling numbers (up to 40) were tried without positive result. Addition of PCR-enhancing substances – betaine, formamide or di-methyl sulfoxide (Henke et al., 1997; Kovarova and Draber, 2000) – to the reactions did not have an effect. Control reactions (using a plasmid carrying a bacterial amoA gene) were
performed for all reactions and consistently resulted in a PCR product. Amplifications using \textit{cbbL}G- and \textit{cbbLR}-specific primers (Selesi et al., 2005) were performed once (Fig. 1). Cloning and sequencing were performed as recently described (Juretschko et al., 1998). The sequence diversity within the clone libraries (except for archaeal \textit{amo} genes and \textit{cbbL} genes from the Kamchatkan enrichment culture and amplifications including primer Nbur1148 for the Buryatian culture which were picked randomly) was screened via RFLP using the 4-cutter enzymes MspI and AluI or RsaI (Fermentas Life Sciences). From each unique RFLP-pattern at least one representative clone was sequenced.

Nucleic and amino acid sequences were phylogenetically analyzed using the software programs ARB (Ludwig et al., 2004) and PHYLIP (Felsenstein, 1993) using comprehensive databases of the respective molecules. The automatic tools of ARB were used to align sequences to the respective in-house databases, which was followed by a manual refinement of the alignments. A 16S rRNA consensus tree was reconstructed based on Maximum-Likelihood (ML) calculation using the Hasegawa, Kishino and Yano substitution model (Hasegawa et al., 1985). For this analysis only sequences >1,350 nt in length were used and a filter only considering positions that were present in \( \geq 50 \% \) of all proteobacterial sequences in the database was applied. For the analyses of CbbLR protein sequences (210 amino acids), Fitch-Margoliash distance trees (7 jumbles, Kimura substitution model (Kimura, 1980), randomized sequence order) were reconstructed using Phylip without the use of conservation filters. In addition, parsimony bootstrapping (1,000 iterations) analyses were conducted using ARB.

\textit{FISH and CARD-FISH}

To visualize ammonia oxidizers and other microorganisms in the enrichment cultures, FISH and CARD-FISH were performed as described recently (Daims et al., 2005; Hatzenpichler et al., 2008) at hybridization times of 2-4 h using the probes listed in Tab. S2. Two clade-specific (Nmir203 and Nmir1009) and two strain-specific probes (Nbur1130 and Nkam1130) were designed using ARB and successfully applied at formamide concentrations of 20\% (Nmir203 and Nmir1009), and 20-35\% (Nbur1130 and Nkam1130) in the hybridization buffer and appropriate washing buffers. None of these newly designed probes has a full-match to non-target sequences when tested \textit{in silico} via probecheck (Loy et al., 2008) and ARB (Ludwig et al., 2004). In addition, ethanol-fixed samples were hybridized with the newly designed probe Therm830 (Tab. S2) specific for \textit{Thermaerobacter}-related sequences obtained from the Buryatian enrichment (35\% formamide concentration). For CARD-FISH,
fluorescein- and Cy3-labelled tyramides were synthesized according to Pernthaler et al. (Pernthaler et al., 2004) and diluted 1:750 parts in amplification buffer. In addition, tyramides labeled with the Br-containing dye eosin isothiocyanate were purchased (The Midland Certified Reagent Company, Inc.) and used 1:1,000 in amplification buffer. After FISH or CARD-FISH, cells were DAPI-stained (1 µg ml\(^{-1}\)) and microscopically analyzed using a LSM 510 scanning confocal microscope (Zeiss) and the included software. DAPI-fluorescence images were taken on a Zeiss Axioplan 2 epifluorescence microscope using the included AxioVision software (Zeiss).

Quantification of FISH-signals was performed for \(^{14}\text{C}\)bicarbonate-labeled betaproteobacterial cells (see below) using the probe Nbur1130 at a formamide concentration in the hybridization buffer of 20 % and a hybridization time of 3 h. For this purpose, biomass samples from the different incubation experiments were immobilized on the same slide (two slides for the two replicates) to avoid slide to slide variations. The cells were microscopically analyzed and the cells from the second replicate of the experiment with 1 mM ammonium in the medium were found to exhibit the strongest FISH signal intensities (quantified via Daime, (Daims et al., 2006)), which were thus set to 100 % (relative intensity). All other values were calculated as percentages of this maximum signal (Fig. S5). For each experiment and replicate >2,500 cells were analyzed, except for the control experiments lacking ammonium and the dead control which both contained a low cell density (for these experiments ~1,000 cells were measured each).

**Immunofluorescence staining**

During the early stages of enrichment (Fig. 1) FISH was combined with immunofluorescence (IF) staining to test for the presence of ammonia monooxygenase (Amo) enzymes in cells of the Buryatian enrichment culture. The FISH-IF protocol was established using ethanol-fixed *E. coli* BL21 cells heterologously expressing the chlorite dismutase (Cld) protein of “*Candidatus Nitrospira defluvii*” (Maixner et al., 2008). For this experiment, FISH was performed using probe EUB338-I, while IF was made using Cld-targeted primary rabbit antibodies (Maixner et al., 2008) and secondary Fluos-labeled anti-rabbit antibodies. For FISH-IF of potential ammonia-oxidizers, ethanol-fixed cells from the enrichment culture were immobilized on glass slides and hybridized with probes Nkam1130 and EUB338mix (Tab. S2) at 63.5 °C (equivalent to 35% formamide at 46 °C) for 1.5 h. When FISH was performed in the presence of formamide, no IF-signals could be obtained from *E. coli* cells, due to unknown reasons. To circumvent a similar problem for the betaproteobacterial cells, an
increased temperature, which had been proven successful in the FISH-IF-protocol of *E. coli*, was applied. Afterwards, a standard FISH washing step was performed at 48 °C. After the slides had been air-dried, the samples were covered with 0.05% Tween20 (Roth) in 1x PBS to which NaCl had been added yielding a final concentration of 1.35 M (salt was added to stabilize the FISH-probe-target hybrids during the otherwise too stringent IF-protocol) and incubated for 10 min at room temperature. Afterwards, slides were dipped into 1x PBS and blocked for 30 min in a humid chamber using 2% BSA in modified 1x PBS (1.35 M NaCl) at room temperature. After a 1 minute wash step in 1x PBS the cells were incubated in a humid chamber for 14 h at room temperature with rabbit or chicken antibodies directed against either AmoA- or AmoB-proteins (Fiencke and Bock, 2004; Lebedeva et al., 2005) diluted 1:500 in 2% BSA, in modified 1x PBS (1.35 M NaCl). Afterwards, the slides were washed twice in 1x PBS before the cells were incubated in a humid chamber for 30-60 min in the dark at room temperature with secondary antibodies diluted 1:1000 in 2% BSA, 0.9 M NaCl in 1x PBS. The secondary antibodies were Fluos-labeled anti-rabbit and Cy3-labelled anti-chicken antibodies (Dianova) for binding AmoA- and AmoB-directed antibodies, respectively. Reactions were stopped by washing in 1x PBS. DAPI-staining was performed and samples analyzed using a laser scanning confocal microscope (see above).

**Acetylene inhibition tests**

To test for a potential inhibitory effect of acetylene (ethyne, C$_2$H$_2$) on ammonia-oxidation the late Kamchatkan enrichment culture (Fig. 1) was incubated in the presence of 0.025% v/v (25 Pa) acetylene in the headspace. The culture was diluted 1:2 and 1:10 in fresh medium containing 1 mM ammonium and 5 mM bicarbonate (starting pH 7.6) and incubated in 5-10 mL aliquots. Incubations were performed in 100 mL flasks (Schott) that were closed with custom-made caps that enabled regular sampling through small openings in the cover that could be closed with plastic screw caps, 50 mL glass-vials closed with a rubber stopper, and 150 mL flasks (Wheaton Science Products) closed with rubber septa in parallel. As controls, 7 mL aliquots of activated sludge from the waste water treatment plant Ingolstadt (Germany; Tab. S3) were incubated in the presence or absence of acetylene for up to three days at room temperature (~25 °C) in the same flasks. Nitrogen compounds were measured as described above.
Incubation of the Kamchatkan enrichment culture with $^{13}$C bicarbonate for NanoSIMS-analyses

The sensitivity of the culture to centrifugation and other stresses and the sometimes observed very long lag-times after dilution into new medium (see results section) did not allow for a controlled experiment lacking ammonium or $^{12}$C bicarbonate. Thus, to test for autotrophic growth with ammonia as source of energy, the late Kamchatkan enrichment culture (Fig. 1) was incubated in 9 mL aliquots with ammonia and $^{13}$C bicarbonate. For this, 10 vol% of fresh CaCO$_3$-based medium containing 1 mM ammonium were added to an active culture and $^{13}$C bicarbonate (99% $^{13}$C, Cambridge Isotope Laboratories) was added to reach a concentration of 5 mM at pH 7.6-7.7. Closed 100 mL flasks (Schott) were incubated vertically at 46 °C in a heating oven (without shaking). An incubation in CaCl$_2$-based medium was not performed, because much lower ammonia-oxidation rates were observed for this medium. Because of the difficulties with cultivation (described in the results section) a daily sampling for nitrogen compounds was not possible, since it would have led to an escape of volatile substances (including $^{13}$C bicarbonate). Sampling was performed directly after the addition of $^{13}$C-tracer as well as after three and seven days of incubation. Each time, 20 % of the culture volume was harvested (10 min, 13,000 g) and the biomass was fixed for 1 h in 3 % formaldehyde in 1x PBS at room temperature. The supernatants were stored at -20 °C and nitrogen compounds measured later as described above.

NanoSIMS analyses

CARD-FISH was performed directly on silica wafers as described above using eosin isothiocyanate-conjugated tyramides. The protocol was established using mixed PFA-fixed pure cultures of *Escherichia coli* (that had been grown on 100 % $^{13}$C glucose) and *Burkholderia cepacia* and HRP-labeled probes EUB338-I and NonEUB (Tab. S2). Regions of interest were marked using a laser microdissection microscope (LMD7000, Leica) and fluorescence images were taken at the LMD for documentation. Prior to insertion into the NanoSIMS loadlock, wafers were blown off using a high purity N$_2$-jet in order to remove contaminating loose particles.

Analyses were performed on the NanoSIMS 50L device ( Cameca) of the Core Facility for Advanced Isotope Research at the University of Vienna (Austria). Measurement areas were pre-sputtered using high Cs$^+$ beam currents (~175 pA) before measurements were performed. Images were acquired with a 4 pA Cs$^+$ ion primary beam, focused to a spot size of <100 nm. Analyzed regions were 30x30-50x50 µm in area and were rastered using a resolution of
512x512 pixels at a dwell time of ~15 ms. Secondary ions were recorded in the multi-collection mode, with electron multipliers being positioned to detect $^{12}\text{C}$, $^{13}\text{C}$, $^{12}\text{C}^{14}\text{N}$, $^{31}\text{P}$, $^{32}\text{S}$, $^{79}\text{Br}$ and $^{81}\text{Br}$ ions. Secondary electrons were detected simultaneously. To resolve isobaric species (e.g. $^{12}\text{C}^1\text{H}$ vs. $^{13}\text{C}$, $^{12}\text{C}^1\text{H}_2$ vs. $^{14}\text{N}$) a mass resolving power ($\text{M}/\Delta\text{M}$) of ~7,500 was adjusted. Data was analyzed using the Cameca WinImage software. In this analysis, single cells or small clusters of cells (3-5 cells) were defined as regions of interest (ROI; Fig. 3). Total bromine ($^{79}\text{Br}+^{81}\text{Br}$) counts were normalized to the total carbon counts ($^{12}\text{C}+^{13}\text{C}$). For $^{13}\text{C}$-incorporation studies in the enrichment culture ROI were defined by the $^{12}\text{C}^{14}\text{N}^-$ ion image, guided by the identification of target organisms via $^{79}\text{Br}$ and $^{81}\text{Br}$. $^{13}\text{C}$-incorporation values were corrected for the natural background of $^{13}\text{C}$ (1.0837 %; as determined for cells that had been sampled at the start of the experiment) and the dilution of $^{13}\text{C}$-tracer in the [$^{12}\text{C}$]bicarbonate-containing medium (see below).

Measurement of bicarbonate concentrations

Concentrations and ratios of [$^{13}\text{C}$] and [$^{12}\text{C}$]bicarbonate in the culture medium used for NanoSIMS activity tests were measured using GC-IRMS. For this, at the end of the experiment cells were separated from the medium via centrifugation (7 min, 13,000 g) and aliquots of the supernatants were injected into GC-sampling vials containing pure helium gas. The medium was acidified using 85% ortho-phosphoric acid ($\text{H}_3\text{PO}_4$) to outgas CO$_2$. Then, samples (in triplicate) and standards of known concentration (494 ppm and 1020 ppm CO$_2$ at natural $^{13}\text{C}$-concentration, i.e. ~1.08%; in duplicate) were measured using a Thermo Finnigan Delta V Advantage mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled to a Thermo Fisher GasBench II. The extent of equilibration of $^{13}\text{C}$-tracer with $^{12}\text{CO}_2$ in the air before transfer into the vials containing helium gas occurred was tested and found to be negligible. The labeling ratios determined via GC-IRMS were used to calculate total carbon-labeling values for NanoSIMS-experiments in which both $^{13}\text{C}$ and $^{12}\text{C}$ were present in the incubation medium. For this, the $^{13}\text{C}$-values measured by NanoSIMS were multiplied with the correction factor (7.24) obtained via GC-IRMS.

Incubation of the Buryatian enrichment culture with [$^{14}\text{C}$]bicarbonate for FISH-MAR-analyses

For bicarbonate uptake experiments (Fig. 1), the enrichment culture containing the novel betaproteobacterial microbe as well as the known AOA $N$. gargensis (Hatzenpichler et al., 2008) was grown in medium containing CaCl$_2$ (but not CaCO$_3$) until ammonium was
depleted. Then, 2.1 mL aliquots of the culture were transferred into horizontally fixed 10 mL glass vials (Roth). Incubations with different ammonia concentrations (0.1, 1 and 10 mM) were run in parallel (pH ~7.7). In addition, controls without ammonium and with dead cells (PFA-fixed to test for chemography) in the presence of 1 mM ammonium were performed. To test for a potential inhibitory effect of allylthiourea (AITU), controls containing 1 mM ammonium and 100 µM AITU were performed. For each condition two biological replicates were conducted. The cultures were pre-incubated under the respective conditions for 3 h before 3.25 µCi $[^{14}C]$bicarbonate were added to each vial. The vials were placed inside closed 50 mL plastic tubes (aerobic conditions) and incubated at 46°C without shaking. After 16 h, cells were harvested (13,000 g, 10 min), washed with 1x PBS and PFA-fixed as described above. Ammonia depletion was measured photometrically as described above. Unfortunately, due to technical problems no exact nitrite/nitrate data are available for these samples, but the production of nitrite/nitrate was checked via Merckoquant (Merck) test stripes.

**Screening of sludge samples from waste water treatment plants**

For environmental screening, two FISH-probes specific for the novel betaproteobacterial clade (probes Nmir203 and Nmir1009, see Tab. S2) were used to screen PFA-fixed samples from 27 diverse waste water treatment plants (wwtps) located in Austria, Germany, the United Kingdom and Switzerland (Tab. S3). After this initial screening, DNA extracted via a phenol/chloroform-protocol (see above) from sludge of the wwt of the University of Veterinary Medicine Vienna, Austria (UVM Vienna), was used as template for a PCR using clade-specific primers. Nmir203F and Nmir1026R (Tab. S1), identical in sequence to the respective FISH-probes, were unsuccessfully used in different combinations with primers 616V, 630R or 1492R (Lane, 1991; Juretschko, 2000) at different annealing temperatures (48-62 °C), a cycling number of 30-35 and the addition of BSA. In addition, a nested approach, with an initial amplification step using 616V and 1492R (54°C; 30 cycles), and a second PCR (using 616V and Nmir1026R; 30 cycles) in which 1 µl of the PCR-product of the first reaction was used, was unsuccessfully tried.

**Incubation of waste water treatment plant sludge with $[^{14}C]$bicarbonate for FISH-MAR analyses**

Sludge from the wwt of the UVM Vienna was sampled and incubated at 24 °C until ammonium was depleted. Then, sludge was diluted 1:10 in filter-sterilized sludge supernatant and 4.5 mL aliquots were transferred into 50 mL glass vials. Incubations with two different
ammonium concentrations (0.3 and 1 mM) were run in parallel to controls without ammonium and dead cells (PFA-fixed) in the presence of 1 mM ammonium. Controls containing 1 mM ammonium and 100 µM AITU were performed. For each condition two biological replicates were conducted. The cultures were pre-incubated at 20 °C under the respective conditions for 48 h before 10 µCi \[^{14}C\]bicarbonate were added to each vial and ammonium was replenished to the respective intended starting concentration (i.e. 0.3 mM or 1 mM). The vials were closed using rubber stoppers and incubated at 24 °C at slight vertical shaking for 6 h (first sampling) and 22 h (second sampling). At each sampling point 2 mL of sludge were harvested via centrifugation (13,000 g, 10 min), washed twice with 1x PBS and PFA-fixed (as described above). Production of nitrite and nitrate was checked using Merckoquant test stripes (Merck).

**Microautoradiography**

Incubations with radioactively labeled substrate were performed with the Buryatian enrichment culture and sludge from the wwtp of the UVM Vienna (see above). After incubation and fixation, FISH (using strain- and clade-specific probes, respectively) and DAPI-staining was performed with these samples as described above. The hybridized slides were dipped in preheated (48 °C) LM-1 emulsion (Amersham) and exposed for 7-44 days (enrichment culture) or 5-60 days (sludge) at 4 °C in the dark. Then, slides were developed for 5-7 min in Kodak D19 solution (40 g L\(^{-1}\) distilled water), washed (1 min in distilled water, 4°C), fixed for 3 min in sodium-thiosulfate solution (Na\(_2\)S\(_2\)O\(_3\), 300 g L\(^{-1}\) distilled water, 4°C) and again washed (1-2 min in distilled water, 4°C). For each condition and biological replicate, at least two technical replicates (i.e. microautoradiography slides) were microscopically examined.

**Metagenomic sequencing of the Kamchatkan enrichment culture**

At the time of DNA extraction for metagenomic sequencing the AOA “Candidatus Nitrosotenuis uzonensis”(Hatzenpichler et al., in prep.), was still present in the culture (Fig. 1). The culture was grown aerobically in 300 mL Erleyenmeyer flasks as described above. Biomass was harvested (11,000 rpm, 10 min) from 125 flasks and frozen (-20 °C) until phenol/chloroform-based DNA-extraction (see above) was performed. 11.8 µg of DNA was stored in TE-buffer and shipped at ~4 °C to Genoscope. Metagenomic sequencing and partial assembly of the genome of the betaproteobacterial strain was performed by Genoscope (France). DNA was submitted to mechanical fragmentation through nebulization (hydroshear),
with a 3 kb mean fragment size. After recirculization using an adapter, DNA was fragmented using an E210 ultrasound DNA-shearer (Covaris) and 600 bp fragments containing the adapter were selected for sequencing. 3/4 of a 454 Titanium mate-paired run were performed, generating ~250 Mb on 796,942 reads. In addition, Illumina (GA-IIx) single lane sequencing was performed generating 34,689,904 reads, each 36 bp in length. The 454-reads were used for assembly using Newbler, generating 1977 contigs (739 contigs >500 bp) on 41 scaffolds with a cumulative size of ~5.5 Mb for the total metagenome. Illumina reads were used to correct 454 sequencing errors as described by Aury et al. (Aury et al., 2008). Corrected contigs were assembled using the scaffold description and were used to generate the final sequence prior annotation. GC-content plots (Fig. S1) and 16S rRNA gene analysis of the reads suggested the presence of two dominant populations. 112 out of 359 obtained partial 16S rRNA sequences were highly similar to the betaproteobacterial sequences obtained earlier via a PCR-assay. 238 were affiliated to *N. uzonensis*, while the remaining were affiliated to other microbes. All reads assigned to the largest scaffold, totaling 1,649,125 bp on 14 contigs and corresponding to *N. uzonensis* (Hatzenpichler et al., in prep.), were in the following removed from the dataset. The remaining reads were submitted to a second Newbler-assembly that generated 88 scaffolds which were binned using rRNA-markers, GC-content and coverage. The betaproteobacterial draft genome is represented by ~3.48 Mb distributed over 182 contigs on 18 scaffolds and has an average GC-content of 66.7 %. The MaGe software (Vallenet et al., 2006) was used for the prediction of coding sequences and automatic annotation as recently described (Lücke et al., 2010).

**Results**

*Establishment of enrichment cultures*

Two enrichment cultures seeded with material from thermal springs in the Buryatian Republic (Lebedeva et al., 2005; Hatzenpichler et al., 2008) and from the Kamchatka peninsula were established and grown in the lab for nine and four years, respectively (see Fig. 1 for an timetable of experiments). During the initial enrichment phases different incubation temperatures were tested. 46 °C was chosen for incubation of both cultures as this temperature yielded the highest nitrite production rates from ammonium (Lebedeva et al., 2005) (not shown in this manuscript for the Kamchatkan culture). The Buryatian culture during all times of the enrichment contained the group I.1b AOA *N. gargensis* (Hatzenpichler et al., 2008). In the early stages of enrichment the group I.1a AOA *N uzonensis* was found to
be highly enriched in the Kamchatkan culture as demonstrated by the molecular analysis of key gene sequences and CARD-FISH (Hatzenpichler et al., in prep.). With advancing enrichment it was possible to obtain a culture that contained the novel AOA as sole known ammonia oxidizer (Hatzenpichler et al., in prep.). In addition, in a parallel enrichment (Fig. 1), which is described in this manuscript, a betaproteobacterial species was found to be highly enriched, while the AOA was depleted according to CARD-FISH. No differences in ammonia-oxidizing activity could be detected when the two cultures were grown in the presence or absence of the pH-indicator cresol-red (not shown). In later stages (after 2007 and 2008 for the Buryatian and Kamchatkan culture, respectively; not indicated in Fig. 1) cresol was not added as it may provide an additional source of carbon and/or energy.

*Sensitivity of ammonia oxidizers to different stresses*

With proceeding enrichment the cultures displayed high sensitivity to stresses, including centrifugation, lack of substrate (ammonium) in the medium and incubation in glass flasks closed with rubber stoppers. Centrifugation speeds as low as ~5,000 g (5-10 min at 4, 23 or 40 °C) led to a stop of nitrite production after transfer of the centrifuged cells into fresh medium for several weeks. During later stages of the enrichment, lack of ammonium in the enrichment medium even for very short periods of time (1-5 days) led to a stop of ammonia oxidation that persisted for ten days to up to five weeks after addition of new substrate, or inoculation (1:2-1:10) into fresh medium. During lag-times depletion of ammonium without concomitant nitrite production was often (but not always) observed. All these effects were more pronounced in the Kamchatkan culture, which, in addition, repeatedly proved sensitive to different rubber stoppers or septa, resulting in a complete stop or significantly reduced rate of nitrite production. Despite repeated cooking, washing and autoclaving of different stoppers/septa in combination with different glass bottles/flasks, the culture proved to be sensitive to all tested setups tested except the described Schott flasks approach (see materials and methods). Due to these reasons control incubations in medium deplete of either \[^{13}C\]bicarbonate or ammonium could not be performed, because every single (of many) attempt(s) (centrifugation or lack of substrate in the medium) led to a long-term (3-5 weeks) shut-down of ammonia oxidation. Furthermore, these effects impeded us from studying the effect of acetylene on the Kamchatkan enrichment culture, because all flasks that could have been used for such incubations proved to be inhibitory to the culture, or could not be closed properly to hinder gas escape during sampling (as was the case for Schott and Erlenmeyer flasks).
Screening for known AOA and AOB and identification of a single dominant bacterial phylotype in the two enrichment cultures

PCR-based screening for known AOB in the two enrichment cultures was performed as recently described (Hatzenpichler et al., 2008), but no amplification product using different primer sets specific for bacterial amoA genes could be obtained (Fig. 1). In addition, for both cultures several 16S rRNA gene clone libraries were established using general bacterial primers, but of the retrieved sequences none was related to known AOB. In total ~300 and 50 16S rRNA gene fragment clones were screened and assigned to 5 and 3 different RFLP-patterns for the Buryatian and Kamchatkan culture, respectively. Taken together 75 clones from both enrichments representing all RFLP-types were sequenced in full length (additional clones were partially sequenced) and phylogenetically analyzed (Fig. 2). All sequences obtained were highly similar to each other (≥99.3 %, respectively) and formed a monophyletic group within the beta-subclass of Proteobacteria together with a single environmental clone sequence (accession nr. AF407711, with 93.9% similarity to the species from the Buryatian thermal spring). Only moderate similarities (<94% identity) to a few other sequences from public databases were found.

The sequences obtained from the Buryatian culture were highly similar (>99.7%) to 16S rRNA fragments recently obtained from the same enrichment containing the AOA N. gargensis (Hatzenpichler et al., 2008). For this culture, an additional PCR was performed to check for potential chimera-formation. It could be demonstrated that partial 16S rRNA sequences amplified via a clone-specific primer-pair are virtually identical to the sequences amplified via general bacterial primers (data not shown), rendering the origin of the obtained sequences from chimera formation during PCR unlikely. Furthermore, a metagenomic sequencing dataset of a parallel Buryatian culture (Fig. 1) highly enriched in N. gargensis (Zumbraegel et al., unpubl.) contained four partial 16S rRNA-sequences (221-1,100 nucleotides in length, >97-100% similarity to clone library sequences) of the novel betaproteobacterial strains. Unfortunately, all sequences were encoded on small contigs (max. ~1,800 nucleotides), impeding a more detailed analysis of the organism’s genome. Furthermore, all other bacterial marker genes in this data set that could have been used for phylogenetic binning (e.g. ribosomal proteins) were also located on small contigs (all <1,100 nt). In addition to the betaproteobacterial 16S rRNA sequences, one partial 23S rRNA (~1,100 nt in length) and three partial 16S rRNA gene fragments (175-400 nt in length), all affiliated to the gram-positive, strictly aerobic, heterotrophic Thermaerobacter genus, could
be identified in the *N. gargensis* metagenomic data set. The presence of *Thermaerobacter* in the Buryatian enrichment culture could be validated by PCR using the “inhibitory primer” Nbur1148lock, which hampers amplification of 16S rRNA genes of the novel betaproteobacteria. From 20 randomly sequenced clones from this experiment, only 9 still exhibited high similarity to the betaproteobacterial sequences obtained using general PCR-primers. The other sequences were related to *T. subterraneus* and related species (88-89 % sequence similarity) and were highly similar (97-99 %) to the sequences obtained via metagenomics. A low abundance (<1 % according to visual inspection) of *Thermoaerobacter*-related straight to slightly curved rods (data not shown) in the Buryatian enrichment culture could be demonstrated using the specific FISH-probe Therm830 (Tab. S3). The observed morphology is consistent with the ones of cultured representatives, such as *T. marianensis* (Takai and Horikoshi, 1999).

The inability to obtain bacterial *amoA* gene sequences (Fig. 1), encoding the catalytically active subunit of ammonia monooxygenase, and 16S rRNA gene sequences of known bacterial ammonia oxidizers from both enrichment cultures is consistent with the inability to detect known AOB in these cultures using the AOB specific FISH-probes listed in Tab. S2. Archaeal 16S rRNA and *amoA* gene amplification products could be obtained during all stages of the enrichment from the Buryatian culture (Fig. 1). While archaeal sequences could be detected in earlier stages of the Kamchatkan enrichment, the subculture established in mid 2010 (Fig. 1) was dominated by the novel betaproteobacterium (Fig. 4) while archaia could not be detected by neither 16S rRNA- or *amoA*-targeted PCR nor via CARD-FISH (Fig. 1). The sequences obtained from the former culture were identical to those of the ammonia-oxidizing thaumarchaeote *N. gargensis* (Hatzenpichler et al., 2008; Spang et al., 2010). However at a later stage during the Kamchatkan enrichment (Fig. 1), when cells were exposed to [13C]bicarbonate (see below) archaeal *amoA* but not archaeal 16S rRNA gene sequences could be detected by PCR if cells were directly added to the PCR reaction (either undiluted or diluted 1:10 in sterile water). Controls using environmental DNA (extracted from activated sludge of the Humber waste water treatment plant that contains *amoA*-encoding archaia; see Mußmann *et al.*, in review) and the above archaia-specific primers persistently yielded positive PCR-results. The same result was observed when lysed cells from the Kamchatkan enrichment were used in a PCR with general bacterial 16S rRNA gene primers (Fig. 1).

*cbbl* gene sequences, coding for the large-subunit of bacterial red-like ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), were PCR-amplified from both cultures (Fig. 1), while genes coding for green-like RuBisCO (*cbblG*) could not be detected. 2 and 4
cbbLR clones were sequenced from the Buryatian and Kamchatkan culture, respectively. The sequences were related (>90 % amino acid identity) to RuBisCOs of Bradyrhizobium sp. and Acidithiobacillus sp., respectively, and exhibited moderate similarity (87-91 % amino acid identity) to sequences of known AOB (Fig. S2).

FISH-detection of AOA and novel betaproteobacteria
In the Kamchatkan enrichment no archaeal cells could be detected by CARD-FISH using published probes (Arch915, Cren512, RHGA702; see Tab. S2) for several months (late 2010 to 2011; Fig. 1) and, thus, several culture transfers. In contrast, *N. gargensis* (detected using probes Arch915 or RHGA702) was permanently present in the Buryatian enrichment (Fig. 1). Species- (Nbur1130 and Nkam1130) and genus-specific (Nmir203 and Nmir1009) FISH-probes (Tab. S2) for the novel betaproteobacterial phylotypes were designed and successfully applied to the enrichment cultures. With the exception of the general bacterial probe EUB338-I, no other of the tested FISH-probes including the *Betaproteobacteria*-specific 23S rRNA-targeted probe Bet42a (Tab. S2) hybridized to cells identified via the newly designed probes. In both cultures, all cells detected by the respective strain-specific probe also hybridized to both genus-specific probes, with probe Nmir1009 resulting in a much brighter fluorescence signal than probe Nmir203. In all FISH-hybridizations of the two enrichment cultures targeting the novel betaproteobacteria, Cy3- and Cy5-labeled probes yielded much stronger fluorescent signals than Fluos-labeled probes, which often were hardly detectable at all. Although different aliquots and dye-labels were tested, EUB338-I consistently yielded lower fluorescent signals for the novel betaproteobacteria than the newly designed specific probes. This difference was not observed when CARD-FISH using fluorescine-labelled tyramides was performed. Generally, cells detected via probe Nbur1130 in the Buryatian enrichment were rods of 1.7±0.3 µm in length and 0.9±0.1 in width (n=35 cells from MAR-experiment with 1 mM NH₄Cl; Fig. 1 and S4) and constituted 20-50 % of all DAPI-stained cells at the different stages of enrichment (see also Hatzenpichler et al., 2008). Differences in cell morphology affecting length and diameter could be observed during different stages of the enrichment (data not shown). Cells detected via probe Nkam1130 in the Kamchatkan culture typically were smaller with a length of 1.5±0.2 µm and a diameter of 0.6±0.1 µm (n=35, measured for cells of the ^13C-incubation experiment; Fig. 1) and constituted at least 90% of DAPI-stained cells during the late stages of the enrichment (Fig. 4). It is possible that cell morphology of the betaproteobacteria was slightly altered because cell dimension
measurements where performed after fixation and FISH. No unspecific labeling of cells was observed in any sample using control probes (Tab. S2).

**Immunofluorescence detection of Amo-proteins**
A FISH-immunofluorescence (FISH-IF) protocol was successfully established and optimized using *E. coli* BL21 cells heterologously expressing the chlorite dismutase (Cld) enzyme of *N. defluvii* (Maixner et al., 2008). However, not all *E. coli* cells detected by FISH gave a signal with IF, as can be seen in Fig. S3A. This can probably be explained by cell-to-cell differences in the heterologous expression of *Nitrospira*-proteins in *E. coli*. Hybridization of the antibody to *E. coli* BL21 cells that did not express Cld did not yield a IF-signal. No FISH-signal was obtained when the control probe NONEUB (Tab. S2) was used for FISH-IF-labelling of *E. coli* BL21 cells expressing Cld (not shown).

In the early stages of the ammonia-oxidizing enrichment from the Buryatian thermal spring (Fig. 1), FISH-IF targeted at bacterial AmoA- and AmoB-proteins was performed using recently described antibodies (Fiencke and Bock, 2004; Lebedeva et al., 2005). While it was not possible to demonstrate the presence of AmoA-proteins, AmoB-antibodies repeatedly labelled cells hybridized to the species-specific probe Nbur1130 (Fig. S3B). However, other bacterial cells (DAPI and EUB338mix-positive) which were not detected by probe Nbur1130 also yielded a positive IF-signal, which could be caused by unspecific antibody staining or the presence of another bacterial species expressing an ammonia monooxygenase (Fig. S3B). Unfortunately, it was not possible to perform additional control experiments, because soon after the experiments described above had been performed the AmoB-targeted antibody that we obtained from our collaborators at the University of Hamburg, was not available any more. The identity and potential for ammonia oxidation of the unidentified bacteria remains unknown, because these cells (or better, their morphotype as observed by FISH and DAPI-staining) were lost from the enrichment culture shortly after the IF-experiments. No unspecific labeling of enrichment culture cells was observed when control FISH-probes (Tab. S2) or Cld-targeted antibodies (see above) were applied, demonstrating the general suitability of the newly developed FISH-IF protocol.

**CO$_2$-incorporating activity of novel betaproteobacteria in the Kamchatkan enrichment culture**
Incubation of the enrichment culture in the presence of ammonia and [${}^{12}$C]/[${}^{13}$C]bicarbonate was performed in duplicate (Fig. 1). Due to the described difficulties encountered during
cultivation (rubber stopper/septa-enclosed flaks that would have allowed easy sampling could not be used; see above), the first flask was sampled only at the end of the experiment (after seven days of incubation). Until that time only a minute amount of the ammonia in the medium had been oxidized to nitrite (~35 µM). The second flask was sampled (and thus opened) twice: first after three days and another time at the end of the experiment. After seven days ammonia had nearly stochiometrically been oxidized to nitrite (an increase of ~700 µM nitrite and decrease of ~800 µM ammonia was measured). However, the highest activity was observed in the first three days after which already 63 % of the total ammonia depletion/nitrite production could be demonstrated.

To directly link the identity of the organisms of interest with their potential carbon-fixing activity a halogen in situ hybridization (HISH)-NanoSIMS protocol, similar to that recently described by others (Behrens et al., 2008; Musat et al., 2008), was developed. The method which leads to bromine deposition in CARD-FISH stained cells via the use of eosine-labeled tyramides, was established using mixtures of pure cultures and successfully applied to samples of the Kamchatkan enrichment culture. On average 5-10 times higher bromine counts (normalized to total carbon counts) were detected for ROI using specific probes compared to a control probe (NonEUB) for both pure culture mixes (not shown) and enrichment culture cells (Fig. 3EF). For enrichment culture cells, differences in the relative bromine ion counts were observed between individual target cells. Similar variations in signal intensity were also detected when conventional fluorescently labelled tyramides were used for CARD-FISH and probably reflect differences in ribosome content or cell wall permeability.

The background of $^{13}$C in enrichment culture cells was determined by analyzing the isotope’s abundance in single cells sampled at the start of the experiment (Fig. 3A) and found to be almost equal to the natural $^{13}$C abundance (~1.08 %). After seven days of incubation, but low ammonia-oxidizing activity, the betaproteobacterial cells of the first replicate flask demonstrated an average $^{13}$C-labelling of 4.41 % (after correction for dilution of $^{13}$C in $^{12}$Cbicarbonate-containing medium; see material and methods) (Fig. 3B). No significant difference in $^{13}$C-label of betaproteobacteria was observed for the two time points analyzed for the second replicate (3.00 and 3.08 % label for three and seven days of incubation, respectively; Fig. 3CD). However, for two target cells, a high $^{13}$C-labelling of 58.44 % clearly indicative of autotrophic activity could be observed in the three day sample (Fig. 3C). In some samples carbonaceous material (detection of both $^{13}$C- and $^{12}$C-) was present on the wafers (Fig. 3BD). This material did not contain $^{79}$Br/$^{81}$Br, $^{14}$N, $^{31}$P or $^{32}$S and most probably represents residual precipitated carbonate from the medium. All cells analyzed via HISH-
SIMS were affiliated to the novel identified betaproteobacterial strain (as evidenced by a specific oligonucleotide probe), demonstrating the organisms’ numerical dominance in the culture.

**CO₂-incorporating activity of novel betaproteobacteria in the Buryatian enrichment culture**

In a previous publication, the Buryatian betaproteobacterium was found to be co-enriched with the AOA *N. gargensis*, but at this time no indication for an involvement of the former microbe in ammonia oxidation was found (Hatzenpichler et al., 2008) (Fig. 1). Later exposure to radiographic emulsion of cells from the same [¹⁴C]bicarbonate-experiment for 1.5-2 months suggested a potential minor involvement of these cells in ammonia oxidation based on the assimilation of radioactive tracer (not shown).

In a new attempt (Fig. 1), the enrichment culture containing both the novel betaproteobacterial species and *N. gargensis* was incubated in the absence or presence of ammonia and with [¹⁴C]bicarbonate as sole sources of energy and carbon, respectively. Different concentrations of ammonia (~0.1 mM, 1 mM and 10 mM free ammonia after pre-incubation) were tested in duplicate. For all concentrations most, but not all, novel betaproteobacterial cells showed MAR-signals. The intensity of the MAR-signal (silver grains were not quantified) was directly correlated with the increase in ammonia availability in the medium (Fig. S4). No MAR-labeling could be observed in the controls that either (i) lacked ammonium (Fig. S4) or (ii) contained dead cells (not shown). In most incubations containing ammonium the AOA was MAR-labeled and, thus, actively participated in the oxidation of ammonia. Exceptions are both replicates with 0.1 mM ammonia in the medium, in which AOB, but not no AOA, were slightly radioactively labeled. For unknown reasons, in one of the duplicate incubations with 10 mM ammonia no nitrite production was observed. Consistently, no cells were radioactively labeled when tested with MAR (exposition of slides for up to 44 days; not shown). Addition of 100 µM of the inhibitor allylthiourea (AITU) to an incubation with 1 mM ammonia did not yield a noticeable reduction of MAR-signals above betaproteobacterial or AOA cells (not shown). Due to technical problems no precise nitrite data are available for this experiment. However, nitrite was shown to be produced in all relevant incubation setups (except in one of the 10 mM setups where also no MAR-labeling could be observed; see above) using test stripes. Nitrate was undetectable during the whole experiment (Merckoquant test stripes).
Quantification of FISH-signal intensity of the betaproteobacteria in dependence on the concentration of ammonia

FISH was performed using probe Nbur1130 with cells that had been incubated under different ammonia concentrations during the MAR-experiment described above. Identical experimental conditions were used in all experiments to guarantee direct comparability of FISH-signals. However, no clear correlation between the FISH signal intensity reflecting the cellular ribosome content in dependence on the ammonia-concentration was observable (Fig. S5).

Environmental screening for the novel betaproteobacteria

Fixed sludge samples from 27 different WWTPs across Europe (Tab. S3) were analyzed for the presence of the novel betaproteobacteria using probes Nmir203 and Nmir1009 (Tab. S2). While all samples were tested negative using probe Nmir203, four sludge samples (two from WWTP Lyss, and one from plant Kraftisried and the plant of the UVM Vienna) comprised colonies of Nmir1009 (and EUB338mix) positive cells (Tab. S3). However, using DNA extracted from the Viennese treatment plant sludge as template, primers identical in sequence to the above FISH-probes in different combinations with general bacterial primers (including a nested PCR-approach) did not yield an amplification product. However, control PCR-reactions using a plasmid carrying the 16S rRNA gene of the novel Buryatian microbe spiked to the sludge DNA prior to PCR were positive.

The potential for ammonia-oxidizing activity of the Nmir1009-positive cells in sludge from the UVM Vienna WWTP was tested by FISH-MAR. Sludge biomass was pre-incubated in the absence or presence (0.3 mM or 1 mM) of ammonium before radioactively labeled bicarbonate was added to test for autotrophic growth in dependence of ammonium. Under ammonia-oxidizing conditions, known betaproteobacterial AOB (for applied FISH-probes see Tab. S2) were strongly labelled at both substrate concentrations (not shown), while MAR-positive Nmir1009-labelled colonies were never observed (after up to two months of exposition of MAR-slides). In incubations with 1 mM ammonium and 100 µM allylthiourea (AITU), a known inhibitor of bacterial Amo (Hooper and Terry, 1973; Ginestet et al., 1998), nitrite/nitrate production ceased and neither MAR-positive AOB- nor Nmir1009-colonies were observed (data not shown).
**Preliminary insights into the genome of the Kamchatkan betaproteobacterium**

The genome of the novel betaproteobacterium enriched from a Kamchatkan thermal spring was partially reconstructed using an environmental genomics approach shortly before the completion of my PhD-thesis. Therefore, only a very preliminary analysis can be presented here. The draft genome is represented by ~3.48 Mb distributed over 182 contigs on 18 scaffolds, with an average GC-content of 66.7 %. Other features of the genome are listed in Tab. 1.

The 16S rRNA gene encoded in the obtained genome is highly similar to sequences from previous PCR-assays (>$99.6 \%$ identity), demonstrating that at least closely related strains were present during the whole enrichment. Consistent with the negative results from FISH (see above), the binding site on the 23S rRNA of the studied microbe exhibits 11 mismatches to probe Bet42a. The limited coverage of this (86 \% group coverage according to Amann and Fuchs, 2008) and other phylum- or subphylum probes has recently been discussed (Loy et al., 2003; Loy et al., 2007; Amann and Fuchs, 2008; Barr et al., 2010).

Complete Calvin-Benson-Basham (Calvin) and tricarboxylic acid (TCA) cycles as well as a complete glycolysis pathway and the entire set of genes (NADH-dehydrogenase, succinate dehydrogenase, cytochrome bc1 complex, cytochrome c oxidase and a F-type ATPase) essential for oxidative phosphorylation are encoded in the genome. The organism seems not to be capable to fix CO$_2$ via a reversed TCA-cycle, because both key enzymes (2-oxoglutarate synthase and ATP-citrate lyase) are not encoded. Surprisingly, the $cbbL$ gene sequence, encoding the large subunit of RubisCO, of the sequenced organism has only moderate similarity (90.7 and 93.8 \% identity on nucleotide and amino acid level, respectively) to the ones previously obtained via clone libraries. These gene fragments, however, formed a monophyletic group with the sequence obtained via the metagenomic approach.

No homologues of either copper- (including archaean and bacterial particulate ammonia or methane monooxygenases, Amo or pMmo) or iron-dependent monooxygenases (soluble methane monooxygenase, sMMO) or hydroxylamine (Hao), hydrazine (Hzo) or nitrite (Nxr) oxidoreductases could be found in the partial genome sequence. However, the organism has the genetic potential for denitrification of nitrate to nitrous oxide (N$_2$O) via nitrite and nitric oxide (NO), and is able to reduce nitrite to ammonia via an NADH-dependent assimilatory nitrite reductase (EC 1.7.1.4). Furthermore, transporters for ammonium, nitrate, formate/nitrite, glutamate/aspartate, mannitol, amino acids and di/oligopeptides are present in the genome.
Discussion

Two moderately thermophilic members of a novel clade within the beta-subclass of *Proteobacteria* were enriched with ammonia as only substrate in the growth medium from two geographically distinct Russian thermal springs. At the moment, no closely affiliated cultured representatives of these organisms are available and 16S rRNA gene sequences similar to the ones of the enriched bacteria have only very scarcely been reported from environmental samples (Fig. 2). Both microbes were originally found in considerable numbers in ammonia-oxidizing enrichment cultures containing AOA (Hatzenpichler et al., 2008 and Hatzenpichler *et al*., in prep.). The Buryatian species was already identified in a co-enrichment with the group I.1b AOA *N. gargensis* in a former study (Hatzenpichler *et al*., 2008). However, no indication for a potential involvement of this betaproteobacterium in ammonia oxidation was found at that time, despite cells that had been incubated in the presence of ammonia and [14C]bicarbonate were exposed to radiographic emulsion for up to 14 days (Hatzenpichler *et al*., 2008). However, later, exposure of cells from the same experiment to radiographic emulsion for 1.5-2 months resulted in some weak signals which could indicate a potential involvement of these cells in ammonia oxidation, heterotrophic assimilation of carbon dioxide (Roslev et al., 2004; Hesselsoe et al., 2005) or cross-feeding between the AOA and these bacteria. The other novel betaproteobacterial species has been identified in a culture seeded with material from a thermal spring in the Uzon caldera and initially had been found to be co-enriched with the group I.1a AOA *N. uzonensis* (Hatzenpichler *et al*., in prep.). During later stages of the enrichment the AOA (Fig. 1) was not detectable any more by CARD-FISH and PCR. However, we cannot exclude that even at this stage of enrichment very few AOA cells were still present in the enrichment. Consistent with this hypothesis, amoA gene sequences affiliated to *N. uzonensis* (but no archaeal 16S rRNA genes) were detectable by PCR if cells from the [13C]bicarbonate-labelling NanoSIMS-experiment were directly used for PCR although the enrichment was clearly CARD-FISH-negative for archaea.

HISH-SIMS experiments were performed to demonstrate the potential of the Kamchatkan betaproteobacterium to grow autotrophically under ammonia-oxidizing conditions. While autotrophic growth could be demonstrated for a few cells (Fig. 3CE), most cells exhibited only low carbon fixation activity (Fig. 3CD). This assimilation was in its extent indistinguishable from that of cells grown in a parallel culture that showed for unknown reasons very low ammonia oxidizing activity. This result suggests that the majority of the betaproteobacterial cells were not involved in ammonia oxidation and that, possibly, the
carbon assimilation in these cells was due to the activity of cellular carboxylases. In light of the very recently obtained genomic data (see below) such processes are the most plausible explanation for the observed $^{13}$C-content of most of the HISH-identified cells. It has been reported, that 1.4-6.5 % of cellular carbon is derived from such heterotrophic carbon dioxide assimilation processes (Roslev et al., 2004; Hesselsoe et al., 2005). These values are well in range of the measured $^{13}$C/$^{12}$C-ratios of most cells analyzed in this study. Yet, the high $^{13}$C-enrichment of two analyzed cells can only be explained by autotrophic growth and suggests a physiological micro-heterogeneity of the studied microbes. Autotrophic growth is consistent with the early finding of proteobacterial $cbbL$ gene fragments, encoding the large subunit of RubisCO, in the culture and the presence of the complete Calvin-cycle in the genome of the Kamchatkan betaproteobacterium. Another possibility is that the majority of the betaproteobacterial cells were actually engaged in the oxidation of ammonia, but that the obtained energy, due to unknown reasons, could not be used to build-up new biomass. Interestingly, no cells other than the novel betaproteobacterium identified by a specific HISH-probe demonstrated assimilation of $^{13}$C-tracer. This view may, however, be biased, as the studied betaproteobacterium made up >90% of all cells of the enrichment culture (Fig. 4). Thus, it is possible that a small population of other autotrophically growing cells are present in the culture, but has been overlooked due to the small number of fields of view which could be analyzed by NanoSIMS.

A new set of FISH-MAR experiments of the Buryatian culture performed after the publication of Hatzenpichler et al. 2008 also suggested the ability of the novel betaproteobacterium to grow autotrophically (Fig. S4). Given the applied experimental conditions the MAR-labelling was too intense to be explained via heterotrophic fixation of carbon dioxide (Roslev et al., 2004; Hesselsoe et al., 2005). This is in agreement with the PCR-based detection of proteobacterial RubisCO-sequences in this enrichment (Fig. S2). In addition to the betaproteobacteria, intense MAR-labelling of *N. gargensis* cells could be observed (data not shown). Most interestingly however, in both duplicate setups carried out in the presence of 0.1 mM ammonia in the medium, a part of the betaproteobacterial population was slightly MAR-positive, while AOA cells did not exhibit any label. This is in apparent contrast to the recent observation that *N. gargensis* is highly active in the presence of 0.14 mM ammonia (Hatzenpichler et al., 2008). It must however be noted, that *N. gargensis* may behave inconsistently at different stages of enrichment (e.g. by selection of different strains at different time-points) and that other factors, such as co-enriched species, might also influence the results in non-axenic cultures. Furthermore, with these enrichments apparently stochastic
variations occur even in replicate experiments possibly caused by minor contamination of the media or the glassware (also see below). For example, in one (of a duplicate) incubation performed in the presence of 10 mM ammonia no substrate depletion/nitrite production or MAR-labelled cells could be observed at all, while the other replicate was highly active. AlTU, a known inhibitor of bacterial Amo, did not have a pronounced effect on the carbon-fixation activity of either *N. gargensis* or the betaproteobacterium. The finding that the AOA is not affected by this inhibitor is consistent with the study of Hatzenpichler *et al.* who reported that AlTU led to a decrease but not an inhibition of its metabolic activity (Hatzenpichler *et al.*, 2008). Interestingly, the FISH-signal intensity and, thus, the ribosome content of the novel betaproteobacterium does not correlate with ammonia concentration or carbon-fixation activity (Fig. S5). A similar situation has been observed for AOB which were reported to have high ribosome contents even after long episodes of physiological inhibition or starvation (Wagner *et al.*, 1995; Morgenroth *et al.*, 2000).

According to the automatic genome annotation, the Kamchatkan betaproteobacterium is genetically capable of autotrophic growth, but also encodes pathways consistent with heterotrophy/mixotrophy. Complete Calvin- and TCA-cycles as well as complete gene sets for glycolysis- and oxidative phosphorylation as well as a range of transporters involved in the import of amino acids, di- and oligopeptides, formate/nitrite, glutamate/aspartate and mannitol are encoded in the genome. Interestingly, a set of denitrification genes that enable the organism to gain energy from the reduction of nitrate to nitrous oxide via nitrite and nitric oxide are encoded in the genome. A similar situation is observed in many AOB that engage in “nitrifier denitrification”, a detoxification process that reduces nitrite to nitrous oxide (Lipschultz *et al.*, 1981; Klotz and Stein, 2008). Most striking, however, is the absence of homologues of *amoABC* and *hao* genes, which was already suggested by the inability to detect *amoA*-genes via established PCR-assays. It is possible that the respective genes exhibit mismatches to the primers used and in addition were missed during sequencing. However, the completeness or near-completeness of several metabolic pathways and the presence of central information processing genes (*e.g.* encoding DNA- or RNA-polymerases and tRNAs for all amino acids; not shown) indicate that the partial genome is almost complete.

In order to directly link the identity of microbes to the potential presence of proteins in their cells, a FISH-IF protocol was developed. It could be shown that the highly enriched betaproteobacterial cells in the Buryatian culture bind AmoB-antibodies (Fig. S3), but fail to yield a positive signal with antibodies specific for AmoA. Unfortunately, cells other than the studied betaproteobacteria were also detected by the AmoB-targeted antibody, drawing into
question the specificity of the antibody used. These observations, together with the absence of amo-like genes in the genome of the highly related Kamchatkan species, an unspecific binding of the polyclonal AmoB-antibodies in our hands (Fiencke and Bock, 2004; Lebedeva et al., 2005) appears as the most plausible explanation for these results.

Both cultures, most importantly the one derived from the Kamchatkan thermal spring, repeatedly proved sensitive to the absence of ammonia in the growth medium. This high sensitivity to substrate unavailability is in strong contrast to reports from known AOB. For both Nitrosomonas europaea (Tappe et al., 1999) and Nitrosospira briensis (Bollmann et al., 2005) very short lag-times of a few minutes to hours were observed even after extended periods (up to 3 months) of starvation. This for the best part is explained by the inherent stability of the Amo-protein under physiological conditions (Pinck et al., 2001) and the constantly high cellular ribosome content of AOB (Wagner et al., 1995; Morgenroth et al., 2000). These features are generally interpreted as adaptations of these organisms to the temporally fluctuating substrate concentrations in the environment (Stein and Arp, 1998; Tappe et al., 1999; Koops et al., 2003; Bollmann et al., 2005; Bock and Wagner, 2006). Such adaptations would, however, not have evolved (or passed on during many generations) if the respective group of organisms lived in a habitat with a constant supply of substrate. Whether this is the case for the thermal springs from which the seeding material for the described enrichment cultures had been obtained cannot be answered, because no data on the temporal fluctuation of ammonia or other substances in these habitats are available.

Starvation for a few days followed by transfer into fresh medium led to lag-times of the novel betaproteobacteria (or other unknown ammonia oxidizers in the enrichment) of 2-5 weeks, during which nitrite production was never observed, although in some cases ammonium depletion was detectable. This is consistent with the observation that starved AOB actively take up ammonia/ammonium and store it intracellularly reaching cytoplasmic concentrations of 0.8-1 M (Schmidt et al., 2004; Weidinger et al., 2007). Furthermore, putative transporters for ammonia/ammonium are encoded in the genomes of all sequenced AOA (Hallam et al., 2006; Walker et al., 2010; Blainey et al., 2011; Hatzenpichler et al., in prep; Zumbraegel et al., in prep.) as well as the Kamchatkan betaproteobacterium.

In addition, centrifugation proved to be a major stress for both cultures. This may be explained by the presence of only a very thin peptidoglycan layer in both betaproteobacteria as observed by transmission electron microscopy (Elena Lebedeva and Eva Spieck, personal communication). This might contribute to the organisms’ sensitivity to osmotic and similar stresses and the observed polymorphism of the Buryatian cells.
To test for the environmental distribution of the newly discovered betaproteobacterial clade a diverse range of 27 waste water treatment plant (wwtp) sludges was screened with newly designed specific FISH-probes (Tab. S2), and 4 sludge samples were tested positive (Tab. S3). Sludge from the wwtp of the Viennese University of Veterinary Medicine was screened more thoroughly. However, no evidence for the presence of the novel bacterial genus or the ammonia-oxidizing activity of FISH-identified cell colonies could be obtained via PCR and FISH-MAR, respectively.

The near-complete absence of 16S rRNA sequences (Fig. 2) closely related (i.e. \( >90 \% \) similarity) to the newly discovered clade of betaproteobacteria from public databases (Genbank and Camera, (Sun et al., 2011)) indicates that these organisms may be confined to only a small number of understudied habitats (e.g. thermal springs). Another possibility may be that these microbes are present in the environment only in small abundances, which makes them hard to be detected by traditional clone library-based studies or untargeted metagenomics.

In summary, after over five years of research the following alternative explanations for the physiology of the novel betaproteobacterial clade remain:

a. the microbes are lithotrophic organisms that live from the oxidation of ammonia, the only (purposely added) exogenous source of energy in the enrichments. This idea is supported by the apparent absence of any known ammonia oxidizer during the final stages of one of the enrichments, the observation of a near-stoichiometric formation of nitrite under ammonia-depleting conditions (while nitrate remains undetectable) in enrichments dominated by the betaproteobacteria (\( >90 \% \) abundance), and the clear autotrophic lifestyle of a small part of the betaproteobacterial population. However, no copper-containing monooxygenases, that in other microbes are responsible for the aerobic oxidation of ammonia, could be found in the incomplete genome of the Kamchatkan betaproteobacterium. Furthermore, gene fragments encoding ammonia-oxidizing enzymes could not be amplified from the two enrichments using established PCR-assays. Allylthiourea, a known inhibitor of bacterial (Hooper and Terry, 1973; Ginestet et al., 1998), but not archaeal (Hatzenpichler et al., 2008), ammonia oxidation had no effect on the nitrifying activity of the Buryatian co-enrichment.

The only possibility we can currently think of by which the organisms could potentially engage in the oxidation of ammonia is if they could reverse the reaction of the cytoplasmatic NADH-dependent assimilatory nitrite reductase that organisms typically use to grow on nitrite/nitrate as sole source of nitrogen and that is encoded in the genome of the Kamchatkan
betaproteobacterium. This hypothesis, however, is not supported by the literature and, without further experimental evidence, remains purely speculative.

b. The newly identified microbes are heterotrophic organisms that live from either aerobic respiration or microaerobic denitrification with oxygen or nitrate/nitrite as final electron acceptor, respectively, but are nevertheless capable of carbon-fixation. The presence of sets of genes for oxidative phosphorylation and near-canonical denitrification in the partial genome support these hypotheses. In conflict to denitrification, however, is the observation that nitrite accumulated almost stoichiometrically to the depletion of ammonia in the enrichment culture and nitrate could not be detected. Furthermore, the cultures were grown under fully aerobic conditions (Hatzenpichler et al., 2008), though micro-aerobic niches might have existed on the micron-scale level. However, it should be noted that measurements of gaseous nitrogen compounds have not been performed, raising the possibility that small amounts of nitrite are actually reduced in the growing enrichment. As observed for the Kamchatkan betaproteobacterium, some heterotrophic bacteria, such as *Methylococcus capsulatus* (strain Bath) or “*Candidatus Accumulibacter phosphatis*”, encode key genes for carbon-fixation (Ward et al., 2004; Garcia Martin et al., 2006). However, the latter organism was not reported to actively express these genes *in situ* (He et al., 2010; He and McMahon, 2011).

The hypothesis of an unknown organic substance fuelling the growth of the highly abundant betaproteobacteria is supported by the low but detectable uptake of $^{13}$C-tracer by the Kamchatkan betaproteobacteria under conditions in which only minute production of nitrite from ammonia could be observed. On the other hand, when the Buryatian culture was incubated in the presence of 10 mM ammonium and $[^{14}$C]bicarbonate, in one assay betaproteobacteria and *N. gargensis* were MAR-negative even after prolonged exposition of the slides (up to 44 days). Consistently, no nitrite-production was observed in this experiment when tested by nitrite-sensitive stripes. However, other factors may have caused this result. Exogenous sources for organic substrates by contamination of the anorganic medium seem unlikely, because the enrichments have been grown in the same media in three geographically distant laboratories (located in Hamburg, Moscow and Vienna) with different water sources, suppliers of chemicals, and glass ware. Because growth under ammonia-oxidizing conditions was also observed in flasks closed by plastic or aluminium caps, contamination from stoppers or septa can be excluded as relevant sources of contamination. Despite the possibility that organic substrate was provided to the betaproteobacteria by co-enriched microbes this hypothesis raises many questions. Ammonia is the only source of...
energy in both enrichments. Thus, an ammonia oxidizer must form the basis of the food chain, providing all other organisms in the enrichment with their respective sources of energy. However, known AOA and AOB must have been (if at all) present in numbers below the detection limit of standard PCR assays at the later stages of the Kamchatkan enrichment and it is hard to imagine how such a low abundant population could cause the numerical dominance of the betaproteobacteria by cross feeding of metabolic waste-products or by providing nutrients via cell lysis.

In conclusion, additional experiments are required to more thoroughly evaluate the above described physiological scenarios. The recently gained insights from genome sequencing of the novel betaproteobacteria, most importantly their apparent capability for denitrification and indications for the potential use of heterotrophic substrates, could provide important clues for the isolation of these microbes in pure culture, a much needed prerequisite to solve the riddle of the physiology of these unusual organisms.

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**Figure legends**

Fig. 1. Schematic overview of experiments carried out with the Kamchatkan (A) and Buryatian (B) enrichment culture containing a novel clade of betaproteobacteria. Red colors and black dots denote the result and approximate timing of each respective experiment. Abbreviations: AOA, the ammonia-oxidizing archaeon *N. uzonensis* (A) or *N. gargensis* (B); AOB, known beta- and gammaproteobacterial ammonia-oxidizing bacteria; Betas, newly identified betaproteobacteria; +, present/detected; –, absent/not detected; for other abbreviations please refer to the main text. *, while AOA could not be detected via CARD-FISH or 16S rRNA-PCR in the samples incubated in the presence of $^{13}$C-tracer, an *amoA* gene PCR of the same sample yielded a positive result. Dashed boxes indicate the publications associated with these enrichment cultures.

Fig. 2. 16S rRNA-based phylogenetic tree showing the affiliation of the two novel betaproteobacterial strains potentially involved in ammonia oxidation (red) to known AOB (gold). The tree shown is based on a maximum likelihood (ML) calculation and nodes with parsimony bootstrapping support >90 % (1,000 iterations) are indicated by white dots. Numbers in parentheses give the number of sequences within a group. The scale bar equals 10 % estimated sequence divergence.

Fig. 3. Incorporation of $^{13}$C-inorganic carbon by the novel betaproteobacteria in the Kamchatkan ammonia-oxidizing enrichment culture as analyzed by NanoSIMS. Cells of interest were identified by HISH-SIMS using the species-specific probe Nkam1130 (Tab. S2). The relative contribution of $^{13}$C to total carbon is shown for cells that have been incubated for three and seven days in the presence of $[^{13,12}$C]bicarbonate. (A) a sample was taken at the start of the incubation to analyze the background of $^{13}$C in cells of interest and found to represent the natural concentration of this isotope in nature (*i.e.* ~1.08 %). (B) a culture that demonstrated very low ammonia-oxidizing activity was sampled after seven days of incubation. All cells demonstrated a low enrichment in $^{13}$C as compared to the control (4.41 %). (C-D) a highly active culture that exhibited nearly stochiometric depletion of ammonia and formation of nitrite was sampled after three (C) and seven (D) days of incubation with $[^{13}$C]bicarbonate. At both time points, the HISH-identified cells exhibited only a small amount of label (~3.0 %), except two cells that demonstrate a high incorporation (58.44 %) of $^{13}$C (C). For the HISH-image corresponding to (C) please refer to panel (E) of this figure. For (A-D) images on the left side indicate relative $^{13}$C-values, while on the right
side the corresponding $^{12}$C-$^{14}$N$^{-}$ pictures are given. On the right side regions of interest that were used for calculation of $^{13}$C/($^{12}$C+$^{13}$C) and ($^{79}$Br+$^{81}$Br)/($^{12}$C+$^{13}$C) are indicated in red. Note the different scaling for left-side images for (A) and (B-D). (E) betaproteobacteria of the Kamchatkan enrichment culture (also see panel C) are detected using the specific probe Nkam1130 while other cells were visualized via DAPI-staining (not shown). (F) The same sample hybridized with the control probe NonEUB. Bromine counts are normalized to total carbon counts. HISH-signals of specific probes were 5-8 times higher than the background observed in samples hybridized to the control probe. All fields of view are 40x40 µm in size and were measured under identical conditions. Red arrows and white circles indicate cells of high $^{13}$C-content and precipitated carbonate (see text), respectively. Scale bars equal 5 µm.

Fig. 4. Fluorescence microscopic images of a representative field of view demonstrating the abundance of the novel betaproteobacteria in a sample of the Kamchatkan enrichment culture that had been incubated in the presence of $[^{13}$C]bicarbonate. The HISH-SIMS analysis of this sample is presented in Fig. 3C and 3E. (A) DAPI-staining of cells, and visualization of bacteria (B) detected via the general probe EUBmix and the novel betaproteobacteria (C) via the newly designed, strain-specific probe Nkam1130 (Tab. S2). Scale bars equal 10 µm. The DAPI-image has been modified in brightness and contrast to enhance visibility of cells.

Fig. S1. GC-content plots of all contigs obtained via metagenomic sequencing of the Kamchatkan enrichment culture. Each symbol represents a single contig. Contigs associated with the highly covered scaffolds of the AOA N. uzonensis (Hatzenpichler et al., in prep.) and the betaproteobacterium are indicated by squares and circles, respectively. Triangles and crosses denote other contigs/read. The upper and lower panel indicate the size and coverage of each contig as compared to its average GC-content.

Fig. S2. Evolutionary distance (Fitch) tree showing the phylogenetic positioning of RuBisCO-gene fragments obtained from the Buryatian and Kamchatkan enrichment cultures (red) and their relationship to sequences from known AOB (gold). Dots indicate nodes with parsimony bootstrap support ≥90 % (1,000 iterations). Numbers in parentheses denote the number of sequences within a group.

Fig. S3. Results of FISH-IF experiments with pure cultures and the Buryatian ammonia-oxidizing enrichment culture. (A) E. coli cells heterologously expressing the Cld-protein
of “Candidatus Nitrospira defluvii” (Maixner et al., 2008) were first hybridized with FISH probe EUB338-I (red), before Cld-targeted IF (green) took place. In the right picture, FISH was overlayed with IF-signals. (B) FISH-detection of bacteria (EUBmix, blue) and the enriched betaproteobacterial species (Nbur1130, green) was followed by IF-labelling of AmoB-proteins (red). (C) FISH-detection of bacteria (green) and the novel betaproteobacteria (blue) and AmoB-targeted IF-signals (red) are shown. Although cells of the novel betaproteobacterial species consistently bound AmoB-antibodies, other cells (as labeled with DAPI (not shown) and EUBmix) were also immunofluorescently labeled. Whether this was a result of unspecific binding of the antibodies or due to the presence of an additional unknown ammonia-oxidizer unfortunately cannot be answered (see discussion in main text). For a list of FISH-probes refer to Tab. S2.

Fig. S4. Combination of FISH and MAR for visualization of autotrophy of Buryatian enrichment culture cells in dependence of ammonia. Results after incubation with \([^{14}\text{C}]\text{bicarbonate}\) and different ammonia concentrations are shown. Results for seven days of exposition of the MAR-slides are shown for the same replicate. Experimental conditions and microscopic settings were exactly the same for all experiments. Free ammonia concentrations are indicated (starting pH 7.7; 46 °C). Without addition of ammonium to the medium, no MAR signals could be observed (black spots are unspecific background) and cell numbers were very low. The biomass used for these analyses contained in addition to the betaproteobacteria (identified using probe Nbur1130; Tab. S2) and heterotrophic contaminants also the AOA N. gargensis (not shown), which was also participating in the oxidation of ammonia and was labeled in most, but not all, MAR-experiments. The scale bar is the same for all images and indicates ~20 µm.

Fig. S5. Relative FISH-signal intensity of the novel betaproteobacterium enriched from a Buryatian thermal spring in dependence on the concentration of ammonia during the MAR-experiment (see also Fig. S5). Experimental conditions and microscopic settings were identical for the experiments shown. Signal intensity of the incubation with 1 mM free ammonia (second replicate) was found to be highest and thus set as maximum reference value. No clear trend of signal intensity in dependence of ammonia-concentration was observable.
References


Winogradsky, S. (1892) Contributions a la morphologie des organismes de la nitrification. *Arch Sci Biol (St Petersbourg)* **1**: 88-137.


Chapter VI
A. Kamchatkan enrichment

Fig. 1

Sampling

archaeal & bacterial 16S rRNA PCR
AOA +, AOB –, Betas +
archaeal & bacterial amoA PCR
AOA +, AOB –

archaeal & bacterial 16S rRNA PCR
AOA +, AOB –, Betas +
archaeal & bacterial amoA PCR
AOA +, AOB –

Sampling for metagenomics
AOA +, AOB –, Betas +

Acetylene inhibition tests

[13C]-incubation & HISH-SIMS labelling: some Betas +
archaeal 16S rRNA PCR –
archaeal amoA PCR +
cell length measurement of Betas

Betas +
AOA –*, AOB –

AOA +
AOB –, Betas –

Hatzenpichler et al., in prep.

FISH in
regular
intervals
AOA +
AOB –
Betas –

2007

2008

2009

2010

2011
B. Buryatian enrichment

Sampling

archaeal and bacterial 16S rRNA
AOA +, AOB –, Betas +
archaeal and bacterial amoA
AOA +, AOB –

AmoA-targeted IF –
AmoB-targeted IF
Betas +, other cells +

Sampling for metagenomics
AOA +, AOB –, Betas +

archaeal and bacterial 16S rRNA
AOA +, Betas +
archaeal and bacterial amoA
AOA +, AOB –
bacterial cbbL +

1st [$^{14}$C]-incubation & FISH-MAR
labelling: AOA +, Betas –

FISH-signal intensity comparisons of Betas
cell length measurement of Betas

2nd [$^{14}$C]-incubation & FISH-MAR
AOA +, Betas +

Betas +, AOA +, AOB –
Fig. 2
Fig. 3
$^{13}\text{C} \cdot / (^{12}\text{C} \cdot + ^{13}\text{C} \cdot)$

$^{12}\text{C}^{14}\text{N} \cdot$

Fig. 3
\[ \frac{(79\text{Br}^- + 81\text{Br}^-)}{(12\text{C}^- + 13\text{C}^-)} \]

**Fig. 3**
Tab. 1. Overview of key genome features of the novel betaproteobacterium enriched from a Kamchatkan thermal spring.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Value</th>
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<tbody>
<tr>
<td>Partial genome size</td>
<td>3,481,937 bp</td>
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<tr>
<td>Average GC-content</td>
<td>66.7 %</td>
</tr>
<tr>
<td>Number of scaffolds</td>
<td>18</td>
</tr>
<tr>
<td>Number of contigs</td>
<td>182</td>
</tr>
<tr>
<td>Number of genomic objects (CDS, tRNAs, rRNAs)</td>
<td>3,691</td>
</tr>
<tr>
<td>Number of CDS</td>
<td>3,639</td>
</tr>
<tr>
<td>Number of CDS with predicted function</td>
<td>736 (20.2 %)</td>
</tr>
<tr>
<td>CDS density</td>
<td>1.04 CDS/kb</td>
</tr>
<tr>
<td>Average CDS length</td>
<td>883.3 bp</td>
</tr>
<tr>
<td>Average intergenic length</td>
<td>112.5 bp</td>
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<tr>
<td>Protein coding density</td>
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<tr>
<td>Number of 16S-23S-5S rRNA operons</td>
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</tr>
<tr>
<td>Number of tRNAs</td>
<td>44</td>
</tr>
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</table>

CDS, predicted coding sequences
Fig. S1
Buryatian betaproteobacterium

Bradyrhizobium sp., YP_001238690

Nitrosomonas, Nitrosospira, Nitrosococcus (4)

Nitrococcus mobilis, ZP_01125685

Mud sediment, AAX21137

Kamchatkan betaproteobacterium

Acidithiobacillus sp., ACZ9479

Nitrobacter (2)

Rhodospirillum centenum, YP_002300212

Nitrosomonas and Nitrosococcus (4)

10%

Fig. S2
Fig. S4
Fig. S5
Tab. S1. Oligonucleotide primers used for PCR.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5'-3')</th>
<th>Target gene</th>
<th>Specificity</th>
<th>Annealing temperature [°C]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>616V</td>
<td>AGA GTT TGA TYM TGG CTC</td>
<td>16S rRNA</td>
<td>Bacteria</td>
<td>54</td>
<td>(Juretschko et al., 1998)</td>
</tr>
<tr>
<td>630R</td>
<td>CAK AAA GGA GGT GAT CC</td>
<td>16S rRNA</td>
<td>Bacteria</td>
<td>54</td>
<td>(Juretschko et al., 1998)</td>
</tr>
<tr>
<td>1492R</td>
<td>GGY TAC CTT GTT ACG ACT T</td>
<td>16S rRNA</td>
<td>Bacteria and Archaea</td>
<td>54-56</td>
<td>(Lane, 1991)</td>
</tr>
<tr>
<td>Nbur447</td>
<td>GAC GAA ATC GCC CGC ATG</td>
<td>16S rRNA</td>
<td>Buryatian betaproteobacterium</td>
<td>61</td>
<td>this study</td>
</tr>
<tr>
<td>Nbur1148</td>
<td>AGT GCC CAC CTC TCG CGT</td>
<td>16S rRNA</td>
<td>Buryatian betaproteobacterium</td>
<td>61</td>
<td>this study</td>
</tr>
<tr>
<td>Nmir203F</td>
<td>GGA CCT GAT GAG GGC CTC</td>
<td>16S rRNA</td>
<td>novel betaproteobacterial clade</td>
<td>48-56</td>
<td>this study</td>
</tr>
<tr>
<td>Nmir1026R</td>
<td>CAC TCC CCC GTC TCC GGG</td>
<td>16S rRNA</td>
<td>novel betaproteobacterial clade</td>
<td>48-56</td>
<td>this study</td>
</tr>
<tr>
<td>amoA1F</td>
<td>GGG GTT TCT ACT GGT GGT</td>
<td>amoA</td>
<td>bacterial ammonia-oxidizers</td>
<td>48-54</td>
<td>(Rotthauwe et al., 1997)</td>
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<td>amoA2R</td>
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<td>bacterial ammonia-oxidizers</td>
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<td>amoA</td>
<td>bacterial ammonia-oxidizers</td>
<td>48-54</td>
<td>(Holmes et al., 1995)</td>
</tr>
<tr>
<td>A682R</td>
<td>GAA SGC NGA GAA GAA SGC</td>
<td>amoA</td>
<td>bacterial ammonia-oxidizers</td>
<td>48-54</td>
<td>(Holmes et al., 1995)</td>
</tr>
<tr>
<td>Arch-amoAF</td>
<td>STA ATG GTC TGG CTT AGA CG</td>
<td>amoA</td>
<td>archael ammonia-oxidizers</td>
<td>54</td>
<td>(Francis et al., 2005)</td>
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<tr>
<td>Arch-amoAR</td>
<td>GCG GCC ATC CAT CTG TAT GT</td>
<td>amoA</td>
<td>archael ammonia-oxidizers</td>
<td>54</td>
<td>(Francis et al., 2005)</td>
</tr>
<tr>
<td>cbbLG1F</td>
<td>GGC AAC GTG TGT GGS TCC AA</td>
<td>cbbLG</td>
<td>green-like Rubisco type I</td>
<td>62</td>
<td>(Selesi et al., 2005)</td>
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<tr>
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<td>TTT ATC TCT TCC CAC GTT TTC</td>
<td>cbbLG</td>
<td>green-like Rubisco type I</td>
<td>62</td>
<td>(Selesi et al., 2005)</td>
</tr>
<tr>
<td>cbbLR1F</td>
<td>AAG GAY GAC GAG AAC ATC</td>
<td>cbbLR</td>
<td>red-like Rubisco type I</td>
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<td>(Selesi et al., 2005)</td>
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<tr>
<td>cbbLG1R</td>
<td>TCG GTC GGS GTG TAG TTG AA</td>
<td>cbbLR</td>
<td>red-like Rubisco type I</td>
<td>57</td>
<td>(Selesi et al., 2005)</td>
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</tbody>
</table>

References


**Tab. S2.** Oligonucleotide probes used for FISH and/or CARD-FISH.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence (5'-3')</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arch915</td>
<td>GTG CTC CCC CGC CAA TTC CT</td>
<td>Archaea</td>
<td>(Stahl and Amann, 1991)</td>
</tr>
<tr>
<td>Bet42a</td>
<td>GCC TCC CCA CTT CGT TT</td>
<td>Betaproteobacteria</td>
<td>(Manz et al., 1992)</td>
</tr>
<tr>
<td>cluster6a192</td>
<td>CTT TCG ATC CCC TAC TTT CC</td>
<td><em>Nitrosomonas oligotropha</em> lineage (cluster 6a)</td>
<td>(Adamczyk et al., 2003)</td>
</tr>
<tr>
<td>cluster6a192comp</td>
<td>CTT TCG ATC CCC TGC TTC C</td>
<td>competitor for cluster6a192</td>
<td>(Adamczyk et al., 2003)</td>
</tr>
<tr>
<td>Cren512</td>
<td>CGG CGG CTG ACA CCA G</td>
<td>most <em>Crenarchaeota</em></td>
<td>(Jurgens et al., 2000)</td>
</tr>
<tr>
<td>CTE</td>
<td>TTC CAT CCC CCT CTG CCG</td>
<td>competitor for NEU</td>
<td>(Schleifer et al., 1992)</td>
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<tr>
<td>EUB338</td>
<td>GCT GCC TCC CGT AGG AGT</td>
<td>most <em>Bacteria</em></td>
<td>(Amann et al., 1990)</td>
</tr>
<tr>
<td>EUB338 II</td>
<td>GCA GCC ACC CGT AGG TGT</td>
<td><em>Planctomycetales</em></td>
<td>(Daims et al., 1999)</td>
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<tr>
<td>EUB338 III</td>
<td>GCT GCC ACC CGT AGG TGT</td>
<td><em>Verrucomicrobiales</em></td>
<td>(Daims et al., 1999)</td>
</tr>
<tr>
<td>Gam42a</td>
<td>GCC TCC CCA C AT C GT TT</td>
<td><em>Gammaproteobacteria</em></td>
<td>(Manz et al., 1992)</td>
</tr>
<tr>
<td>Nbur1130</td>
<td>AGT GCC CAC CTC TCG CGT</td>
<td>Buryatian betaproteobacterium</td>
<td>this study</td>
</tr>
<tr>
<td>Ncmob</td>
<td>TCC TCA GAG ACT ACG CGG</td>
<td><em>Nitrosococcus mobilis</em></td>
<td>(Juretschko et al., 1998)</td>
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<tr>
<td>NEU</td>
<td>CCC CTC TGC TGC ACT CTA</td>
<td>most halophilic and halotolerant <em>Nitrosomonas</em> spp.</td>
<td>(Wagner et al., 1995)</td>
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<tr>
<td>NIT3</td>
<td>CCT GTG CTC CAT GCT CCG</td>
<td><em>Nitrobacter</em> spp.</td>
<td>(Wagner et al., 1996)</td>
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<tr>
<td>NIT3comp</td>
<td>CCT GTG CTC CAG GCT CCG</td>
<td>competitor for NIT3</td>
<td>(Wagner et al., 1996)</td>
</tr>
<tr>
<td>Nkami1130</td>
<td>AGT GCC CAC TCA TCG CGT</td>
<td>Kamchatkan betaproteobacterium</td>
<td>this study</td>
</tr>
<tr>
<td>Nmir1009</td>
<td>CAC TCC CCC GTG TCC GCC</td>
<td>novel betaproteobacterial clade</td>
<td>this study</td>
</tr>
<tr>
<td>Nmir203</td>
<td>GAG GCC CTC ATC AGG TCC</td>
<td>novel betaproteobacterial clade</td>
<td>this study</td>
</tr>
<tr>
<td>NonEUB</td>
<td>ACT CCT ACG GGA GGC AGC</td>
<td>test for unspecific binding</td>
<td>(Wallner et al., 1993)</td>
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<tr>
<td>Nsm156</td>
<td>TAT TAG CAC ATC TTT CGAT</td>
<td><em>Nitrosomonas</em> spp., <em>Nitrosococcus mobilis</em></td>
<td>(Mobarry et al., 1996)</td>
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<tr>
<td>Nso1225</td>
<td>CGC CAT TGT ATT TGT GA</td>
<td>betaproteobacterial ammonia-oxidizing bacteria</td>
<td>(Mobarry et al., 1996)</td>
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<td>Nso190</td>
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<td>betaproteobacterial ammonia-oxidizing bacteria</td>
<td>(Mobarry et al., 1996)</td>
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<td>Nsv443</td>
<td>CCG TGA CCG TTT CGT TCC G</td>
<td><em>Nitrosospira</em> spp.</td>
<td>(Mobarry et al., 1996)</td>
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<tr>
<td>Ntspa662</td>
<td>GGA ATT CCG CGC TCC TCT</td>
<td>genus <em>Nitrospina</em></td>
<td>(Daims et al., 2001)</td>
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<td>Ntspa662comp</td>
<td>GGA AT CCG CTC TCC TCT</td>
<td>competitor for Ntspa662</td>
<td>(Daims et al., 2001)</td>
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<td>Pla46</td>
<td>GAG TCC CAT CGC TAA TCC</td>
<td><em>Planctomycetales</em></td>
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<tr>
<td>RHGA702</td>
<td>GTG GTC TTC GGT GGA TCA</td>
<td>“<em>Candidatus Nitrososphaera gargarinae</em>”</td>
<td>(Hatzenpichler et al., 2008)</td>
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<td>Therm830</td>
<td>GGT CAA ACC CAC CCA CAC</td>
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<td>this study</td>
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<tr>
<td>Togai424</td>
<td>CTA GCT GCT TCT GGT AGA A</td>
<td>genus <em>Nitrotoga</em></td>
<td>(Lücker, 2010)</td>
</tr>
</tbody>
</table>
References


Tab. S3. Characteristics of screened wwtps. Wwtps labeled in grey were tested positive in FISH-screenings using probe Nmir1009, specific for the novel betaproteobacterial clade. Unfortunately, no specific 16S rRNA gene sequences or evidence for ammonia-oxidizing activity of Nmir1009-cells were found for samples from the wwtp of the University of Veterinary Medicine. DIC, differential internal cycling; na, not available; SBR, sequencing batch reactor

<table>
<thead>
<tr>
<th>WWTP location</th>
<th>Reactor type</th>
<th>Origin of treated sewage</th>
<th>Temp. [°C]</th>
<th>Influent [mg/l]</th>
<th>Effluent [mg/l]</th>
<th>Sampling date</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NH₄⁺</td>
<td>NH₄⁺</td>
<td>NO₂⁻</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NO₂⁻</td>
<td>NO₃⁻</td>
</tr>
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<td></td>
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<td>SBR</td>
<td>municipal, slaughter, dairy waste</td>
<td>13</td>
<td>nd</td>
<td>0.1</td>
<td>0.04</td>
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<td>Bad Zwischenahn, Germany</td>
<td>DIC-SBR</td>
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<td>60</td>
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<td>Bruck an der Leitha, Austria</td>
<td>SBR</td>
<td>municipal</td>
<td>na</td>
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<td>0</td>
<td>na</td>
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<td>na</td>
<td>na</td>
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<td>Hettstedt, Germany</td>
<td>DIC-SBR</td>
<td>municipal and external activated sludge</td>
<td>15</td>
<td>56</td>
<td>12.35</td>
<td>0.24</td>
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<td>Humber, Great Britain</td>
<td>conventional</td>
<td>industrial</td>
<td>26</td>
<td>9</td>
<td>&lt;0.1</td>
<td>na</td>
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<td>Hunzholzen, Germany</td>
<td>DIC-SBR</td>
<td>municipal</td>
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<td>0.03</td>
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<tr>
<td>Ingboldt, Germany</td>
<td>SBR</td>
<td>activated sludge drainage</td>
<td>27</td>
<td>856</td>
<td>0.3</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Kraftisried, Germany</td>
<td>single-stage activated sludge basin</td>
<td>animal rendering</td>
<td>7</td>
<td>397.5</td>
<td>35.3</td>
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<td>industrial (brewery)</td>
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<td>9</td>
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<td>municipal</td>
<td>12</td>
<td>20</td>
<td>1</td>
<td>0.1</td>
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<tr>
<td>Lyss (GZM), Switzerland</td>
<td>membrane filtration plant</td>
<td>animal rendering</td>
<td>30</td>
<td>700</td>
<td>&lt;1</td>
<td>&lt;0.5</td>
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<td>Main treatment plant (HKA),</td>
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<td>municipal and industrial</td>
<td>17.6</td>
<td>23.4</td>
<td>10.8</td>
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<td>fixed bed reactor</td>
<td>animal rendering</td>
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<td>450</td>
<td>&lt;1</td>
<td>&lt;0.5</td>
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<td>OMV (mineral oil company),</td>
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<td>oil refinery</td>
<td>na</td>
<td>na</td>
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<td>na</td>
</tr>
<tr>
<td>Vienna, Austria</td>
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<td>animal rendering</td>
<td>30</td>
<td>750</td>
<td>1</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Plattling, Germany</td>
<td>DIC-SBR</td>
<td>municipal</td>
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<td>na</td>
<td>0</td>
<td>0.05</td>
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<tr>
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Chapter VII

Summary
Zusammenfassung
Summary

Nitrification, the biological aerobic oxidation of ammonia via nitrite to nitrate, is an essential step of the global biogeochemical nitrogen cycle. The recent finding that some archaea, besides two long-known bacterial groups within the beta- and gamma-subclasses of *Proteobacteria*, are capable of ammonia oxidation radically changed our perception of the functional capabilities of members of this domain. Furthermore, it raised fundamental questions regarding the distribution patterns, abundances and activities of the different ammonia-oxidizing lineages in those ecosystem where nitrification occurs. Within the last few years, ammonia-oxidizing archaea (AOA) have been demonstrated to outnumber ammonia-oxidizing bacteria (AOB) in many systems and molecular analyses revealed the presence of a huge diversity of archaeal *amo* genes, encoding the key enzyme ammonia-monooxygenase, in many different environments. However, as monooxygenases of the Amo type are substrate promiscuous it remains to be shown whether the actual substrate of all these archaeal *amoA*-like proteins is ammonia. Furthermore, due to the difficulties of cultivating the slow-growing AOA only a very limited number of enrichment or pure cultures that could be used to elucidate such and other questions regarding the physiology of these organisms are currently available. Interestingly, recent comparative genomics-based inferences of a member of the *amo*-encoding marine group I.1a archaean, “*Candidatus* Cenarchaeum symbiosum”, demonstrated that this organism is profoundly different to the well-established archaeal phyla, and it has thus been proposed that it belongs to a novel phylum named *Thaumarchaeota*. However, as this study was based on only one thaumarchaeotal genome (no more genomes from AOA were available at that time), the scientific community was still reluctant to accept the idea of a novel archaeal phylum.

My Ph.D. studies included five tightly linked projects dealing with ammonia-oxidizing microbes. The core project dealt with the identification of a moderately thermophilic archaean from a hot spring enrichment culture as an ammonia oxidizer and on its initial genetic and physiological characterization. This AOA, provisionally classified as “*Candidatus* Nitrososphaera gargensis”, represents the first cultivated member of the globally distributed soil group I.1b of archaea. It was shown that the metabolic activity of this AOA is influenced by the available ammonia concentration. At low substrate concentrations it is highly active and autotrophically fixes bicarbonate, while slightly elevated ammonia concentrations, known to have no inhibitory effect on any known AOB, lead to inhibition of autotrophy in this
archaeon. The finding that AOA are adapted to low ammonia concentrations was later confirmed and extended by other research groups and at least partially explains the niche differentiation between AOA and AOB.

Based on comparative genomics of *N. gargensis*, *C. symbiosum* and “*Candidatus Nitrosopumilus maritimus*” I contributed to the reinvestigation of the *Thaumarchaeota*-hypothesis. Various genomic features and phylogenetic analyses strongly support both the distinctiveness and ancestral position of the *Thaumarchaeota*. As this phylum might represent the most abundant archaeal lineage on Earth it is of obvious importance to thoroughly study the functional and genetic potential of its members.

The investigation of an enrichment culture seeded with material from a Kamchatkan thermal spring, led to the identification of “*Candidatus Nitrosotenuis uzonensis*”. It represents the first cultured moderately thermophilic AOA affiliated to group I.1a thaumarchaeotes. A near-complete genome of this AOA was obtained via metagenome sequencing and allowed initial insights into the organism’s ammonia oxidation biochemistry and carbon fixation pathway. In addition, it could be demonstrated that this archaeon is a representative of a lineage of AOA globally distributed in terrestrial geothermal environments.

Furthermore, I was involved in a study aimed at elucidating the role of uncultivated thaumarchaeotes in a range of European waste water treatment plants. So far, ammonia oxidation is the only known physiology for this phylum and the term AOA is thus sometimes used as synonym for this lineage. In the presented study it is demonstrated that in one of the analyzed industrial plants in which *amoA*-carrying thaumarchaeotes outnumber AOB by several orders of magnitude, these putative AOA obtain their energy for growth not by ammonia oxidation, but rather by oxidizing yet unknown organic substances. This unexpected finding highlights that archaeal *amoA* genes are an insufficient proxy for detecting and quantifying AOA in the environment and that the Thaumarchaeotes harbour members that use other electron donors than ammonia for energy generation.

In the last project I focussed on the physiological characterization of two novel species of betaproteobacteria that were highly enriched in two ammonia-oxidizing enrichment cultures recently obtained by a collaboration partner from two Russian geothermal springs. In both of these cultures AOA could be demonstrated to contribute to the oxidation of ammonia, but circumstantial data suggested that the betaproteobacteria might also be participating in this process. The near-complete genome of one of these microbes was obtained via metagenomic sequencing and demonstrated that the organism does not encode enzymes known to be involved in the oxidation of ammonia by recognized AOA or AOB. The potential ammonia-
oxidizing activity of these bacteria was tested via the combination of isotope-labelling techniques with *in situ* hybridization techniques. After more than five years of research on this project still no conclusive result can be presented. However, theories on the potential energy conservation pathways of the organisms were developed, which provide a basis for future experiments.
Zusammenfassung


Ein limitierender Faktor ist sicherlich die sehr geringe Zahl der derzeit verfügbaren Anreichungs- bzw. Reinkulturen von AOA, die zur Aufklärung solcher Fragen dienen könnten. Kürzlich zeigten Genom-vergleichende Studien eines „marinen Gruppe I.1a“ Archaeons, „*Candidatus* Cenarchaeum symbiosum“, das dieser Organismus nicht ein Mitglied eines der etablierten Phyla der Archaea ist, sondern tatsächlich in ein neues, bisher unbekanntes Phylum, das nun als *Thaumarchaeota* bezeichnet wird, fällt. Als diese Studie durchgeführt wurde war allerdings nur ein einziges Genom eines Thaumarchaeoten verfügbar, was anfangs zu einer starken Zurückhaltung der Wissenschaftsgemeinde gegenüber der Idee eines neuen Phylums der *Archaea* führte.

Meine Doktorarbeit umfasste fünf thematisch eng miteinander verknüpfte Projekte, die sich mit Ammoniak-oxidierenden Mikroorganismen beschäftigten:

Das Kernprojekt widmete sich der Identifizierung eines moderat thermophilen Archaeons aus einer Anreicherungskultur einer heißen Quelle als Ammoniak-Oxidierer und dessen Charakterisierung. Dieser AOA, einstweilen als „*Candidatus* Nitrososphaera gargensis“ klassifiziert, stellt den ersten kultivierten Vertreter der global verteilten „I.1b Boden-Gruppe“ der *Archaea* dar. Es wurde gezeigt, dass die metabolische Aktivität dieses Archaeons durch die Konzentration des verfügbaren Ammoniaks beeinflusst wird. So ist diese Mikrobe bei niedrigen Ammoniak-Konzentrationen hochaktiv, während sie bereits durch leicht erhöhte Konzentrationen, die noch keinen hemmenden Effekt auf bekannte AOB haben, inhibiert...
wird. Diese Entdeckung, dass AOA an niedrige Ammoniakkonzentrationen angepasst sind, wurde seither durch andere Gruppen bestätigt und erweitert und erklärt zum Teil die Nischen-Differenzierung zwischen AOA und AOB in der Umwelt.


Da dieses Phylum offenbar die zahlenmäßig bedeutendste Linie der Archaea auf unserem Planeten darstellt, fällt dem Studium des funktionellen und genetischen Potentials der Mitglieder dieser Gruppe eine besondere Bedeutung zu.


In meinem letzten Projekt widmete ich mich der physiologischen Charakterisierung zweier neuer Spezies der Beta-Proteobakterien die in zwei Ammoniak-oxidierenden Anreicherungskulturen aus russischen thermalen Quellen stark angereichert sind. Für beide Kulturen konnte gezeigt werden, dass AOA für die Ammoniak-Oxidation verantwortlich
Appendix

Chapter SI

Multicellular photo-magnetotactic bacteria

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and as supplementary online material:
Multicellular photo-magnetotactic bacteria

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Summary
Multicellular magnetotactic bacteria (MMB) are unique microorganisms typically comprised of 10–40 bacterial cells arranged around a central acellular compartment. Their life cycle has no known unicellular stage and division occurs by separation of a single MMB aggregate into two identical offspring. In this study, South-seeking multicellular magnetotactic bacteria (ssMMB) were enriched from a New England salt marsh. When exposed to light, ssMMB reversed their magnetotactic behaviour to become North-seeking. The exposure time needed to generate the reversal response varied with light wavelength and intensity. Extensive exposure to light appeared to be lethal. This is the first report of a Northern hemisphere MMB displaying South-seeking behaviour and the first time a MMB is found to exhibit photo-magnetotaxis. We suggest that this mechanism enables ssMMB to optimize their location with regard to chemical gradients and light intensities, and propose a model to explain the peculiar balance between photo- and magnetotaxis.

Introduction
Multicellular magnetotactic bacteria (MMB) present a life strategy unique in the microbial world. MMB are typically comprised of 10–40 bacterial cells arranged around a central acellular compartment, possibly serving inter-cell communication (Keim et al., 2004a). Every cell is multiply flagellated and contains iron sulphide crystals, mostly greigite (cubic Fe₃S₄). These magnetic particles, known as magnetosomes (for a recent review see Schüler, 2008), are typically located inside invaginations of the magnetotactic bacterial cytoplasmic membrane (Komeili et al., 2006). They are arranged in a way as to orient the bacterial movement parallel to the geomagnetic field lines of the Earth. The life cycle of MMB has no known unicellular stage. Division occurs by separation of a single MMB into two identical offspring (Keim et al., 2004b), while dis-aggregated MMB lose their viability very rapidly (Abreu et al., 2006). While no pure-culture of MMB is currently available, detailed phenotypic characterizations were performed for ‘Candidatus Magnetoglobus multicellularis’ aggregates enriched using magnetic fields (Abreu et al., 2007; Silva et al., 2008; Perantoni et al., 2009). All MMB analysed so far are affiliated by 16S rRNA gene sequence to the delta subclass of Proteobacteria (Abreu et al., 2007; Simmons and Edwards, 2007; Wenter et al., 2009) and recent findings indicate a potential involvement in sulphate reduction (Wenter et al., 2009). Other than a recent report on a chemotactic response of MMB to acetate and propionate (Wenter et al., 2009) potential carbon sources of MMB remain speculative. Very recently, Lefèvre and co-workers reported on the finding of non-magnetotactic multicellular prokaryotes (Lefèvre et al., 2010), indicating more diverse life strategies for this group of organisms then previously assumed.

Magnetotactic bacteria are globally distributed and can account for ~1% of the bacterial population in marine (Flies et al., 2005; Simmons et al., 2007) and freshwater (Spring et al., 1993) habitats. So far, reports of MMB are restricted to marine environments, including hypersaline coastal lagoons (Abreu et al., 2007), salt marshes (Simmons et al., 2007) and tidal sand flats (Wenter et al., 2009). In a hypersaline lagoon and a meromictic salt pond, MMB have been reported to account for 0.001% (Martins et al., 2009) and 1.9% (Simmons et al., 2007) of the total bacterial community respectively. MMB are typically observed in anoxic environments (Abreu et al., 2007) and die rapidly when in the presence of oxygen (Abreu et al., 2006; Martins et al., 2009; Wenter et al., 2009). The genomes of Magnetospirillum magnetotacticum, Magnetospirillum magneticum strain AMB-1 and marine coccus strain MC-1 encode several candidate...
genes for aero-, redox- and potentially photo-taxis (Frankel and Bazylinski, 2009). Motility and positioning mechanisms are expected in magnetotactic bacteria as these organisms seem specially adapted for migration along environmental gradients.

Polar magnetotactic bacteria were thought to be North- or South-seeking in the Northern or Southern hemisphere respectively (Blakemore et al., 1980). However, this concept was challenged when single-celled South-seeking magnetotactic bacteria where reported from a marine basin in Falmouth, MA (Simmons et al., 2006).

Herein, we report on the discovery of South-seeking multicellular magnetotactic bacteria (ssMMB) from a New England salt marsh, exhibiting a combination of photo- and magnetotaxis.

Results and discussion

On-site microscopic analysis demonstrated the dominance of South-seekers (estimated at over 90%) in the MMB population in sediment from a shallow mud pond at the Little Sippewissett salt marsh. Following a week of undisturbed incubation in the lab, ssMMB were enriched to become the dominant magnetotactic organisms in the sample. In a hanging droplet, no change in activity was observed following 2 h of incubation, as opposed to decreased activity after 15–90 min of incubation under similar conditions in previous reports (Abreu et al., 2006; Martins et al., 2009; Wenter et al., 2009). This suggests reduced oxygen sensitivity of ssMMB reported here compared with previously described MMB.

When briefly exposed to UV light (365 nm) at high intensity during observations with a fluorescence microscope, ssMMB changed their magnetotactic behaviour to become North-seeking (Fig. 1; Movie S1), swimming to the opposite side of the droplet. Reversing the magnetic field caused ssMMB to swim back into the light, retaining North-seeking behaviour, with no observed second reversal of swimming direction. Two to five seconds exposure to full-strength UV light at 365 nm appeared to be lethal, with ssMMB losing motility and many becoming disaggregated within minutes following exposure. Heating of the droplet was ruled out as a possible mechanism for the observed response as the light energy was too small to produce a noticeable effect in droplet temperature.

Initial response time as well as the interval between first and last ssMMB to respond (variation) were measured for different light wavelengths and intensities (Table S1). Reversal was observed following 30 s of exposure to approximately 80 W m⁻² of violet-blue light (395–440 nm; Fig. 1; Table S1). The average energy flux of blue light (400–500 nm) at the Earth’s surface at noon, on a clear day, is approximately 170 W m⁻² (Lee and Downum, 1991; Bell et al., 2000), so that this result is well within the intensity values expected in the pond environment.

Fig. 1. Photo-magnetotactic behaviour of ssMMB.
A. ssMMB in a hanging droplet.
B. The same field of view after 20 s of exposure to violet-blue light (395–440 nm) at approximately 410 W m⁻². The black arrow indicates direction of the applied magnetic field.
C. Initial response time as a function of light intensity (395–440 nm). Black line represents best fit logarithmic curve, R² = 0.99.
A stronger influence was observed for shorter wavelengths, with the shortest response time at around 2 s. This response time was measured at medium intensities for UV light (365 nm) or high intensities for longer wavelengths (Table S1). Initial response time appeared to be a function of the natural logarithm of light intensity (Fig. 1C), possibly indicating a cumulative mechanism. No response was observed for wavelengths longer than 490 nm (green), but after 5–10 min exposure to green light ssMMB lost motility, eventually undergoing disaggregation. This observation possibly indicates that the photoresponse mechanism is different from the one responsible for sensitivity to light.

Exposure to blue light (450–490 nm) at approximately 200 W m\(^{-2}\) resulted in a reversible response (Movie S2). MMB switched to North-seeking behaviour but appeared to migrate just outside the intense light in the microscope viewing field. Upon turning the blue light off or switching to longer wavelengths, MMB resumed their South-seeking behaviour. This could be repeated several times before MMB lost their motility, possibly because of accumulated light damage. This observation offers a clue to the ecological significance of the photoresponse mechanism. As light intensities at different depths in the pond are expected to gradually change during the day, this type of balanced response may enable ssMMB to maintain their position within a given light intensity range.

A similar photo response was reported by Frankel and colleagues for the magnetotactic marine coccus strain MC-1, while no such response was observed for *M. magnetotacticum* (Frankel *et al*., 1997). The authors demonstrated that illumination of MC-1 cells with blue (430 nm), yellow (500 nm) or white light triggered a reversible photoresponse, causing the cells to switch their swimming behaviour. This effect was similar to the cell’s response to increased oxygen concentration, overriding their aerotactic response to decreased oxygen concentration (Frankel *et al*., 1997). No reference was made to a lethal effect of illumination on the bacterial population. Recently, it was suggested by Frankel and Bazylinski that an agent of unknown identity obviously prevents South-seeking magnetotactic bacteria in the Northern hemisphere from swimming up to the water surface, because they have not been found in the uppermost water layers (Frankel and Bazylinski, 2009). Based on our findings, we propose that, for some magnetotactic bacteria, light may serve as such a stimulus.

In the Northern hemisphere, a South-seeking magnetotactic organism migrates upwards, towards a more oxygenated layer, in agreement with the apparent low oxygen sensitivity of ssMMB. During daytime, this brings ssMMB towards the sunlit surface. We therefore suggest that the photo-magnetotactic response reported here enables these organisms, for which light appears to be lethal, to optimize their location with regard to chemical gradients and light intensity. Under low-light conditions (e.g. at night) these MMB are thus expected to exhibit South-seeking behaviour, migrating upwards to more favourable conditions. During daytime, MMB reverse their magnetotactic behaviour and seek shelter in the sediment, maintaining their position to avoid excessive light exposure (Fig. 2).

The response described here is different from previously described photophobic behaviour in other bacteria, in which swimming direction is always away from the light. Exposure of ssMMB to light could only trigger the switch from South- to North-seeking polarity, and subsequent exposure did not trigger a switch from North- to South-seeking behaviour. This is appropriate for conditions in their natural environment, where light is always expected to come from above, i.e. from the magnetic South pole. The observed response is most similar to the magnetotactic response of MC-1, with the cells migrating between threshold oxygen concentrations. MC-1 cells respond to light in a fashion similar to ssMMB by reversing their swimming direction (Frankel *et al*., 1997). This could serve as a mechanism to avoid exposure to light when oxygen concentrations in the photic layer are too low to trigger a reversal. However, as ssMMB do not appear to exhibit aerotaxis, they may be migrating between threshold light intensities. ssMMB thus represent an adaptation to life along photon gradients. The advantage derived by ssMMB from migrating towards the water surface, opposite to all previously described MMB, remains to be explored.

One possible explanation for a South-seeking life strategy of MMB on the Northern hemisphere (and vice versa) may be that at the respective sites an inverted redox gradient was present at the time of sampling. This could be, e.g. due to oxygenated fresh water input from an underground reservoir into the more oxygen-depleted salty water. Such a situation could lead to the selection of ssMMB over North-seeking MMB.

The observed response is most similar to the magnetotactic response reported here enables these organisms, for which light appears to be lethal, to optimize their location with regard to chemical gradients and light intensity. Under low-light conditions (e.g. at night) these MMB are thus expected to exhibit South-seeking behaviour, migrating upwards to more favourable conditions. During daytime, MMB reverse their magnetotactic behaviour and seek shelter in the sediment, maintaining their position to avoid excessive light exposure (Fig. 2).

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The discovery of reversal of magnetotactic behaviour in response to a light stimulus in both multicellular (this study) and single-celled magnetotactic bacteria (Frankel et al., 1997; and discussed in Frankel and Bazylinski, 2009) demonstrates this behaviour to be more widespread than previously assumed. The term photomagnetotaxis is proposed to describe this behaviour. The recent report of multicellular non-magnetotactic bacteria exhibiting a similar photoreponse to that reported here (Lefèvre et al., 2010) further supports the existence of multiple life strategies for multicellular bacteria and their apparent sensitivity to light. Further study is needed to find and compare the mechanisms enabling light detection and response of these organisms. It appears now, that some magnetotactic bacteria exercise a fine balance between chemo- and phototaxis to determine their position in environmental gradients. Only by the detailed analysis of these response mechanisms on the genetic, biochemical and phenotypic level will researchers be able to understand how these intriguing microorganisms interact with their complex habitats.

**Experimental procedures**

**Sample collection**

Samples were collected at low tide from 10 different sites at the Little Sippewissett salt marsh, Falmouth, MA. To sample ssMMB, a magnetic stirring bar was placed at the water–sediment interface with the North magnetic pole facing the bucket. The sediment was stirred briefly and allowed to settle for 10–20 min. A small droplet was collected from the point next to the magnetic stirring bar for on-site microscopic analysis. While several samples were tested positive on the presence of MMB, only a sample from a mud pond (coordinates: 41°34′35″N, 70°38′19″W) contained high numbers of these organisms. A 3 l plastic bucket was filled with sediment and water from this pond and maintained at room temperature in the laboratory for 7–14 days.

**Microscopy and photo response**

Magnetotactic bacteria were viewed using the hanging droplet method (Abreu et al., 2006). 4 µl droplets were collected from the point next to the magnet using a pipette and placed on a glass cover slip. The coverslip was inverted and placed atop a small o-ring, and a magnet was placed next to the coverslip. Magnetotactic bacteria accumulated at one side of the droplet, depending on the orientation of the magnet and the magnetotactic behaviour of the bacteria. ssMMB were viewed using a Zeiss Axioscope phase contrast/epifluorescence microscope at 40× magnification. The microscope was equipped with LED white light and mercury arc UV/visible light sources. Cells were exposed to light at different intensities and wavelengths using six filter sets, as detailed in Table S2. Light intensity was controlled using a light attenuation shutter located between the UV/visible light source and the band-pass filter. The shutter had six positions, numbered 1–6, with level 1 being the highest intensity.

**Light intensity measurement**

Light intensity was measured using a LI-COR LI 185B quantum radiometer. The light sensor was covered with a microscope cover slip and placed directly under the 40× objective. Intensity levels 1–5 were measured for filter sets 2–5 as listed in Table S2. Intensity values were corrected according to the instrument manual. Results are detailed in Table S3. Intensity values are given in W m⁻². Measurements for UV light were not performed as the quantum radiometer was unsuitable for measurement of light intensities for wavelengths shorter than 400 nm. However, the light emission...
spectra of the mercury-arc lamp (http://zeiss-campus.magnet.fsu.edu/articles/lightsources/mercuryarc.html) shows similar values for 365 nm and 405 nm, implying similar light intensities for these wavelengths.

**Response time measurement**

Time lapse imaging was used for tracking changes in the number of MMB located at the ‘Southern end’ of a hanging droplet. Images were taken at 0.5–10 s intervals, according to previously observed response times.

**Acknowledgements**

This study was performed during the 2009 Microbial Diversity course at the Marine Biological Laboratory, Woods Hole, MA, USA. The Microbial Diversity course was supported by funding from the US National Science Foundation, the US Department of Energy, The Gordon and Betty Moore Foundation, the Howard Hughes Medical Foundation and the Marine Biological Laboratories. OHS and RH acknowledge financial support by The Gordon and Betty Moore Foundation, the University of Vienna, the Moshe Shilo Memorial Fund, and the Ben Gurion University of the Negev. OHS is recipient of a Levi-Eshkol PhD-fellowship from the Israeli Ministry of Science. RH is recipient of a PhD-fellowship (DOC) of the Austrian Academy of Sciences. OHS and RH acknowledge support by the American Academy of Sciences (USA). The Microbial Diversity course was supported by technical support. We thank Alexander Petroff for helpful discussions, and Zeiss for providing microscopic equipment and teaching assistants (most of all Cristina Moraru) for a great summer. Heather Fullerton is acknowledged for help with sampling. We thank Alexander Petroff for helpful discussions, and Zeiss for providing microscopic equipment and technical support.

**References**


Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Photo response times (s) measured by time lapse image recording.
Table S2. Filter sets used to investigate the phototactic response of South-seeking multicellular magnetotactic bacteria (ssMMB).
Table S3. Light energy flux for different wavelengths and shutter positions (W m\(^{-2}\)).

Movie S1. Photo-magnetotactic response of South-seeking multicellular magnetotactic bacteria (ssMMB).
Movie S2. Reversible photo-magnetotactic response of South-seeking multicellular magnetotactic bacteria (ssMMB).

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Table S1. Photo-response times [sec] measured by time lapse image recording. Initial response presents the time interval after which the first MMB started reversing their swimming behaviour. Variation denotes the interval between initial response and the time of departure of the last MMB. Cells remaining in the microscopic field after this time did not show any movement. These presumably dead MMB made up only a very small portion of the population. This is evident from Figure 1 and Movie 1. No response was observed for wavelengths longer 490 nm. * denote average of two experiments with standard deviation.

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Table S2. Filter sets used to investigate the phototactic response of South-seeking multicellular magnetotactic bacteria (ssMMB).

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Table S3. Light energy flux for different wavelengths and shutter positions [W m\(^{-2}\)].

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NA- not available; energy flux calculated for average wavelength of each filter set.

**Movie 1.** Photo-magnetotactic response of South-seeking multicellular magnetotactic bacteria (ssMMB). ssMMB are crowded at the “South end” of a hanging droplet. Upon exposure to violet-blue light (395-440 nm; after ~1 sec playing time) at approximately 410 W m\(^{-2}\), MMB switch their polarity and migrate towards the “North end” of the droplet. Due to software processing the frame rate of this movie is slightly different from the original recording.

**Movie 2.** Reversible photo-magnetotactic response of South-seeking multicellular magnetotactic bacteria (ssMMB). ssMMB are crowded at the “South” end of a hanging droplet. Upon exposure to blue light (450-490 nm; after ~0.5 sec playing time) at approximately 200 W m\(^{-2}\), MMB switch their polarity and migrate towards the “North end” of the droplet. When blue light is replaced by green light (after ~16 sec playing time), the aggregates return to the “South end” of the droplet. Due to software processing the frame rate of this movie is slightly different from the original recording.
Appendix

Scientific experience

Acknowledgements

Curriculum vitae
Publications in peer reviewed journals
(* equal contribution, § corresponding author)


Manuscripts in preparation or under review


Oral presentations

Talks at scientific conferences


Invited seminars

1. “Candidatus Nitrososphaera gargensis”- A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring (Sept. 15, 2008) Hosted by Dr. Mike Manefield, University of New South Wales, Centre for Marine BioInnovation, Sydney, Australia


3. Nitrososphaera gargensis, Thaumarchaeota and some other hot stuff (May 20, 2010) Hosted by Prof. Victoria Orphan, California Institute of Technology, Geological and Planetary Sciences, Pasadena, USA

Other talks

2. *A heat-loving ammonia-oxidizing crenarchaeote from a hot spring* (Jan. 26, 2008) Yearly workshop of the awardees of the Academy of Sciences. Austrian Academy of Sciences, Vienna, Austria (held in German)

**Conference abstracts**

(* presenting author)


**Teaching experience**

2006, 2010 Advisor of guest scientists at the Department of Microbial Ecology (DOME), University of Vienna (Austria): Dr. Michael Cunliffe (three weeks in 2006), Gilbert Flores (two months in 2008)

2007, 2009, 2010 Supervisor of the two-week undergraduate student course *Fluorescence in-situ hybridization (FISH) - Identification of uncultivated microorganisms*, DOME

2008-2009 Supervisor of a diploma student at DOME: Nathalie Schuster (twelve months)

**Science organization**

The workshop brought together 50 scientists from research institutes in Austria, Denmark, Germany, Great Britain and the USA. During the three-day program 29 talks on different topics in microbial ecology were given.

**Fellowship grants**

2007 Three-year doctoral fellowship (DOC) of the Austrian Academy of Sciences under the project title “Ammonia-oxidation at extreme temperatures: on the origin and limits of nitrification” (90,000 € over three years, 2007-2009)

2011 O. K. Earl postdoctoral fellowship in Geological and Planetary Sciences by the California Institute of Technology, Pasadena, USA. Start of position: summer 2011 (130,000 $ over two years)

**Awards, scholarships & competitive travel grants**

2005-2007 Two travel grants of the European Astrobiology Network Association (EANA) to participate in the annual European Workshop on Astrobiology in Lyon, France (2006) and Turku, Finland (2007) (together 700 €)

2007 Poster award of the Thermophiles conference. Bergen, Norway (250 €)

2008 Travel grant award of the International Society for Microbial Ecology to participate in the 12th International Symposium on Microbial Ecology (ISME) in Cairns, Australia (650 €)

2008 Travel grant of the University of Vienna to participate in the 12th ISME in Cairns, Australia (650 €)
2009 Scholarship award of the Gordon and Betty Moore foundation to participate in the *Microbial diversity course* at the Marine Biological Laboratory (MBL) in Woods Hole, USA (3,500 $)

2009 Scholarship by the University of Vienna to participate in the *Microbial diversity course* at the MBL in Woods Hole, USA (1,000 €)

2009 Scholarship by the Austrian federal state Styria (500 €)

2010 Scholarship of the Gordon and Betty Moore foundation to participate in the *American Society of Microbiology General meeting* in San Diego, USA (~1,200 $)

**Peer review**

since 2009 ad hoc reviewer for the journals *Environmental Science and Technology*, *FEMS Microbiology Ecology* and *The ISME Journal*

**Memberships**

since 2008 International Society for Microbial Ecology (ISME)

since 2009 American Society of Microbiology (ASM)

since 2009 Alumni of the Austrian Academy of Sciences

**Public communication of science**

1. *Microorganisms - the unseen majority* (Nov. 8, 2008) Public talk on environmental microbiology, hosted by the Austrian Academy of Sciences in the course of the *Long night of research*. Vienna, Austria (held in German)

2. *You are never alone!* (Apr. 4, 2009) Public talk on microbial ecology, hosted by the Technical Museum of Vienna. Vienna, Austria (held in German)

   This talk was held as participant and finalist of *Fame Lab Austria 2009*, a national contest on public communication of science organized by the British Council and the organization science2public.
Contributed image


FISH-MAR analyses of a novel member of the *Betaproteobacteria* in an ammonia-oxidizing enrichment culture. The left column displays the fluorescent signals after hybridization of the enrichment with a specific probe for the betaproteobacterium. The middle column shows the corresponding MAR signals and the right column presents an overlay of both figures. Upper panel: Dead control. The cells in the enrichment were killed prior to incubation with $^{14}$CO$_2$ and 1 mM NH$_4^+$ for 16h. No incorporation of $^{14}$CO$_2$ is detectable, excluding chemography or labeling of the cells by the radiotracer in the absence of metabolic activity. Middle panel: The enrichment was incubated with 0.1 mM ammonium in the presence of $^{14}$CO$_2$ for 16h. The cells of the betaproteobacterium are MAR-positive indicating that the bacterium is an autotrophic ammonia oxidizer. Lower panel: Same experiment as in the middle panel but in the presence of 1 mM ammonium. The cells of the betaproteobacterium are strongly labeled demonstrating that this organism fixes more CO$_2$ in the presence of 1 mM than in the presence of 0.1 mM. Scale bar corresponds to 10 µm. It should be noted that in the sample other microbes than the betaproteobacterium were present. These microbes are not visible in the FISH pictures, but could have caused silver grain formation at spots where no cells of the betaproteobacterium were located.
Press coverage

A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring
(Hatzenpichler et al., Proc Natl Acad Sci U.S.A. 105: 2134-2139)

1. Radio interview Ö1 Wissen Aktuell with Prof. Dr. Michael Wagner (07.02.2008)

2. Newspaper Der Standard, print (05.02.2008) and online (07.02.2008): Wiener entdecken neue Mikroorganismen
   http://derstandard.at/3216114

3. Life sciences magazine BIOspektrum (04/2008): Ein thermophiler Crenarchaeot als Ammonium-Oxidierer
   http://www.biospektrum.de/blatt/d_bs_pdf&_id=959851

4. science.orf.at: Wiener Forscher entdeckten neue Mikroorganismen

5. University of Vienna online magazine DieUniversität Online (07.02.2008): Unerwarteter Mikroorganismus in heißer Quelle

6. innovationsreport.de (07.02.2008): Bisher unbekannter Mikroorganismus in heißer Quelle entdeckt

7. chemiereport.de (07.02.2008): Neuer Mikroorganismus in heißer Quelle entdeckt
   http://chemiereport.at/chemiereport/stories/7467/

8. Scinexx - das Wissensmagazin (08.02.2008): Mikrobe in heisser Quelle frisst Ammoniak
   http://www.scinexx.de/wissen-aktuell-7779-2008-02-08.html
Acknowledgements

Most of all I want to thank Michael Wagner for offering me a PhD-position in his department and the support over five years. I have the feeling that from you I have learned all the essentials of our “job”. I admire your dedication to your science and your seemingly inexhaustible knowledge of environmental bugs. Your mentoring and trust in me is greatly acknowledged!

Thanks to Holger Daims for interesting discussions and for having me in his nitrifier-group. Although we sometimes had opposing opinions you always supported me. Thanks for that!

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I am thankful to Christa Schleper for the great collaboration and many interesting discussions over the last years. I very much acknowledge your support for my applications for research fellowships, travel grants and my course participation.

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Thanks go to:
The rest of the DOME-crew - mostly Christiane Dorninger, Alexander (a.k.a. Ralf, Al) Siegl, Elena Tönshoff and “the sugar babes”, Faris Behnam, Jan Dolinsek and Ilias Lagkouvardos, for essential coffee breaks and interesting discussions.

My office-“neighbours” Karin Aistleitner & Astrid Horn for their tolerance ;)

Alexander Galushko, Michael Pester and David Berry for help during culturing and interesting discussions.

Sebastian Lücker for help on how to handle administrative obstacles at the end of my PhD and discussions on microbial physiology and genomics.
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Last, I want to thank my parents whose support brought me to where I am today. For my whole life you encouraged me to do what I am interested in and gave me the opportunities to do so - I can’t thank you enough. This thesis is as much your merit as it is mine!
Curriculum vitae

Roland Stefan Hatzenpichler

Personal information

Date of birth                July 29, 1983
Place of birth              Leoben, Austria
Nationality                 Austrian
Languages                   German (mother tongue), English

Education

Date                      May 2006 - May 2011
Affiliation               Department of Microbial Ecology, University of Vienna, Austria
PhD-thesis                Identification and characterization of novel ammonia-oxidizing archaea and bacteria under the supervision of Prof. Dr. Michael Wagner

Dates                     April 2005 - April 2006
Affiliation               Department of Microbial Ecology, University of Vienna, Austria
Master-thesis             Diversity analyses and in situ detection of nitrifying prokaryotes in hot springs and primeval forest soil under the supervision of Dr. Holger Daims and Prof. Dr. Michael Wagner

Title of qualification    Magister rerum naturalium (Master of natural sciences)

Dates                     October 2001 - April 2006
Affiliation               University of Vienna, Austria
Studies                   Biology, area of focus: microbiology and genetics; including courses in microbial ecology, molecular microbiology, analytical chemistry and organic chemistry
                          completion of studies with distinction

Dates                     September 1993 - June 2001
High school               Bundesgymnasium und Bundesrealgymnasium Leoben, Austria
Title of qualification    high school diploma with distinction
### Additional training

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Participation in the *Microbial Diversity course* led by Prof. Daniel Buckley and Prof. Steven Zinder, which resulted in a publication *(Shapiro et al., 2011)*
Hic sunt dracones

Here be dragons