International Journal of Systematic and Evolutionary Microbiology (2003), **53**, 1485–1494 **DOI** 10.1099/ijs.0.02638-0

16S rRNA and *amoA*-based phylogeny of 12 novel betaproteobacterial ammonia-oxidizing isolates: extension of the dataset and proposal of a new lineage within the nitrosomonads

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The phylogenetic relationship of 12 ammonia-oxidizing isolates (eight nitrosospiras and four nitrosomonads), for which no gene sequence information was available previously, was investigated based on their genes encoding 16S rRNA and the active site subunit of ammonia monooxygenase (AmoA). Almost full-length 16S rRNA gene sequences were determined for the 12 isolates. In addition, 16S rRNA gene sequences of 15 ammonia-oxidizing bacteria (AOB) published previously were completed to allow for a more reliable phylogeny inference of members of this guild. Moreover, sequences of 453 bp fragments of the *amoA* gene were determined from 15 AOB, including the 12 isolates, and completed for 10 additional AOB. 16S rRNA gene and *amoA*-based analyses, including all available sequences of AOB pure cultures, were performed to determine the position of the newly retrieved sequences within the established phylogenetic framework. The resulting 16S rRNA gene and *amoA* tree topologies were similar but not identical and demonstrated a superior resolution of 16S rRNA versus *amoA* analysis. While 11 of the 12 isolates could be assigned to different phylogenetic groups recognized within the betaproteobacterial AOB, the estuarine isolate *Nitrosomonas* sp. Nm143 formed a separate lineage together with three other marine isolates whose 16S rRNA sequences have not been published but have been deposited in public databases. In addition, 17 environmentally retrieved 16S rRNA gene sequences not assigned previously and all originating exclusively from marine or estuarine sites clearly belong to this lineage.

INTRODUCTION

Chemolithoautotrophic ammonia-oxidizing bacteria (AOB) are capable of gaining energy via conversion of ammonia to nitrite and are thus of considerable importance in the global nitrogen cycle. Almost all aerobic environments in which organic matter is mineralized are possible habitats for AOB (Bock & Wagner, 2001). They have been detected in a variety of soil, marine, estuarine and freshwater systems and are crucial for the removal of nitrogen compounds in wastewater treatment plants (Painter, 1986), thus contributing to the impairment of anthropogenic damage to the environment. On the other hand, AOB activity causes deterioration of natural building stones (Bock & Sand, 1993) and enhances nitrogen fertilizer loss from arable soil (MacDonald, 1986). Due to their importance in natural and engineered systems, significant efforts have been made to characterize the diversity, distribution patterns and ecophysiology of AOB (for reviews see Koops & Pommerening-Röser, 2001; Kowalchuk & Stephen, 2001; Koops *et al.*, 2003).

The first isolation of AOB was reported in 1890 (Frankland & Frankland, 1890; Winogradsky, 1890) and since then a considerable number of AOB isolates was obtained from various environments, leading to the description of 16 AOB species (reviewed by Koops *et al.*, 2003). Comparative 16S rRNA gene sequence analyses of these species showed that 'Nitrosococcus halophilus' and *Nitrosococcus oceani* belong to the class '*Gammaproteobacteria*', while the remaining 14

Abbreviation: AOB, ammonia-oxidizing bacteria.

The GenBank accession numbers for the sequences determined in this study are AY123787–AY123813 (16S rRNA gene sequences) and AY123815–AY123840 (*amoA* and AmoA sequences).

Supplementary data are available for 16S rRNA gene sequences and *amoA*/AmoA sequences.

species form a monophyletic lineage within the class '*Betaproteobacteria*' (Head *et al.*, 1993; Pommerening-Röser *et al.*, 1996; Purkhold *et al.*, 2000; Stehr *et al.*, 1995a; Teske *et al.*, 1994; Woese *et al.*, 1984, 1985). Betaproteobacterial AOB encompass the genera *Nitrosomonas* (including 'Nitrosococcus mobilis') and *Nitrosospira* (including *Nitrosolobus* and 'Nitrosovibrio'; Head *et al.*, 1993). Cultured nitrosomonads can be subdivided further into five phylogenetically well-defined lineages (Pommerening-Röser *et al.*, 1996; Purkhold *et al.*, 2000; Stephen *et al.*, 1996). A similar subdivision system has been suggested also for nitrosospiras and was used to assign cultured nitrosospiras into four 'clusters' (Pommerening-Röser *et al.*, 1996; Purkhold *et al.*, 2000; Stephen *et al.*, 1996). However, due to the close phylogenetic relationship of all known nitrosospiras with each other, their subdivision is not well supported by phylogeny inference methods (Purkhold *et al.*, 2000; Koops *et al.*, 2003). The current perception of AOB phylogeny established by comparative 16S rRNA sequence analysis could be confirmed independently by exploiting the gene *amoA*, which encodes the active site subunit of the enzyme ammonia monooxygenase (AmoA), as an alternative phylogenetic marker (Klotz & Norton, 1995; McTavish *et al.*, 1993; Purkhold *et al.*, 2000; Rotthauwe *et al.*, 1995). Generally, 16S rRNA and *amoA*-based trees possess congruent topologies, although the fragment of the latter gene, which is usually used for phylogeny inference, provides less resolution (Koops *et al.*, 2003).

Cultivation-dependent analysis of environmental AOB diversity is time consuming and tedious due to the slow growth rates of these microorganisms. Furthermore, the enrichment and isolation strategies currently applied might fail to recover the entire diversity of this guild. Triggered by these limitations, the last decade saw an enormous increase in molecular, cultivation-independent diversity surveys of AOB. 16S rRNA gene sequences retrieved directly from environmental samples revealed that, with the exception of two lineages within the nitrosomonads (Stephen *et al.*, 1996; de Bie *et al.*, 2001) and one cluster within the nitrosospiras (Stephen *et al.*, 1996), most sequences retrieved environmentally are closely related to cultured AOB (reviewed by Purkhold *et al.*, 2000). Similar findings were obtained by phylogenetic analysis of environmental *amoA* gene fragments (Casciotti & Ward, 2001; Hommes *et al.*, 1998; Klotz & Norton, 1995; McTavish *et al.*, 1993; Purkhold *et al.*, 2000; Rotthauwe *et al.*, 1997; Yamagata *et al.*, 1999).

In the present study, we extended the current 16S rRNA and *amoA* gene databases of AOB by (i) determining the respective sequences of 12 novel AOB isolates and (ii) improving the length and/or quality of several sequences of other AOB published previously. Based on these data, a thorough phylogenetic analysis of betaproteobacterial AOB was performed to obtain a phylogenetic framework, which is required for the design and specificity evaluation of PCR primers and probes and which allows the assignment of environmentally retrieved sequences. Based on the findings obtained, we propose a new lineage within the nitrosomonads which also encompasses many 16S rRNA gene clones from marine systems that were not assigned previously.

METHODS

Pure cultures of AOB. Table 1 summarizes the strains investigated in this study. AOB were cultured using the media and conditions described previously (Koops *et al.*, 1991).

DNA–DNA hybridization. DNA similarities were estimated by photometric determination of thermal renaturation rates, as described by Koops & Harms (1985).

DNA extraction for PCR. AOB were harvested from 10 l of exponentially growing cultures by continuous flow centrifugation (20 000 ***g***, 400 ml min^{-1}). Total genomic DNA was extracted according to the following protocol: a 0·25 g pellet (wet wt) of each sample was resuspended in a 2 ml polypropylene tube containing glass beads (Fast DNA Spin kit for soil; BIO 101) with 500 µl AE buffer (20 mM sodium acetate, 1 mM EDTA, pH 5·5, adjusted with acetic acid), 50 µl 25 % SDS and 600 µl phenol/chloroform/isoamyl alcohol (25 : 24 : 1, by vol.). Cells were lysed in a BeadBeater (BIO 101; 2 × 15 s, speed setting 4·5) and the mixture was then centrifuged (10 min, 10 000 ***g***, 4 °C). The aqueous phase was transferred carefully to a fresh tube, mixed with 600 µl chloroform/isoamyl alcohol (24 : 1, v/v) and centrifuged (10 min, 10 000 ***g***). The aqueous phase was transferred to a fresh tube and, after the addition of 0·1 vol. 3 M sodium acetate, nucleic acids were precipitated by incubation with 0·6 vol. 2-propanol and 5 µl glycogen (5 mg ml^{-1}) for 1 h at −20 °C and subsequently pelleted by centrifugation (20 min, 10 000 ***g***, 4 °C). Pellets were washed with 1 ml ice-cold 70 % ethanol, dried and resuspended in 30–50 µl elution buffer (10 mM Tris/HCl, pH 8·5).

PCR amplification of 16S rDNA. Amplification of 16S rRNA genes was performed as specified by Juretschko *et al.* (1998) and Purkhold *et al.* (2000) using the primers 616F and 630R.

PCR amplification of the *amoA* gene fragment. A 453 bp fragment (without primers) of the *amoA* gene was amplified from 100 ng DNA using the optimized (Stephen *et al.*, 1999) primers *amoA*-1F and *amoA*-2R (Rotthauwe *et al.*, 1997) for PCR with a Primus cycler (MWG Biotech). Reaction mixtures containing 50 pM of each primer were prepared in a total volume of 50 µl using 20 mM MgCl_2 reaction buffer and 1·5 U *Taq* polymerase (Promega). Thermal cycling was carried out by an initial denaturation step at 94 °C for 1 min, followed by 30 cycles of denaturation at 94 °C for 20 s, annealing at 50 °C for 20 s and elongation at 72 °C for 40 s. Cycling was completed by a final elongation step at 72 °C for 5 min.

Cloning, sequencing and phylogeny inference. The amplified 16S rRNA and *amoA* gene fragments were cloned according to the manufacturer's instructions into pCR2.1 TOPO TA vectors (Invitrogen). After plasmid purification (Qiagen), sequences were determined using a Thermo Sequenase Cycle sequencing kit (Amersham), infrared-labelled (IRD 800) primers and an automated DNA sequencer (Li-Cor). 16S rRNA and *amoA* gene sequences were added to the respective database using the ARB program package (http://www.arb-home.de). Sequences were aligned using the package's implemented tools and corrected by visual inspection. Phylogenetic analyses were performed based on nucleic acid (16S rRNA, *amoA*) and amino acid (AmoA) sequences applying distance-matrix (PHYLIP and FITCH), maximum-parsimony and maximum-likelihood methods using the respective tools in the program package. Since the betaproteobacterial AOB encompass a closely related group of microorganisms, no conservation filters were applied and all sequence

Table 1. Pure cultures of AOB used in this study

AOB were obtained from the culture collection of the Institut für Allgemeine Botanik, Abteilung Mikrobiologie, Universität Hamburg, Germany.

Organism	Reference	Origin
Nitrosomonas sp. Nm47	H.-P. Koops (unpublished)	Wastewater treatment plant, Germany
Nitrosomonas eutropha Nm57^T	Koops & Harms (1985)	Wastewater treatment plant, USA
Nitrosomonas sp. Nm58	Stehr *et al.* (1995a)	Sediment, River Elbe, Germany
Nitrosomonas sp. Nm59	S. Sowitzki (unpublished)*	Wastewater treatment plant, Germany
Nitrosomonas sp. Nm84	Stehr *et al.* (1995a)	Suspended particulate matter, River Elbe, Germany
Nitrosomonas sp. Nm86	Stehr *et al.* (1995a)	River Elbe, Germany
Nitrosomonas sp. Nm143	H.-P. Koops (unpublished)	Marine estuary, Dominican Republic
Nitrosomonas sp. Nm148	H.-P. Koops (unpublished)	Hot spring, Santorin, Greece
Nitrosospira sp. Nsp1	Koops & Harms (1985)	Soil, Sardinia, Italy
Nitrosospira sp. Nsp2	Koops & Harms (1985)	Soil, Germany
Nitrosospira sp. Nsp5	Koops & Harms (1985)	Freshwater cave lake, Sardinia, Italy
Nitrosospira briensis Nsp10^T	Koops & Harms (1985)	Soil, Crete
Nitrosospira sp. Nsp12	Koops & Harms (1985)	Soil, Germany
Nitrosospira sp. Nsp17	Koops & Harms (1985)	Soil, Iceland
Nitrosospira sp. Nsp40	H.-P. Koops (unpublished)	Soil, Germany
Nitrosospira sp. Nsp41	H.-P. Koops (unpublished)	Soil, Malta
Nitrosospira sp. Nsp57	E. Spieck (unpublished)*	Masonry, Germany
Nitrosospira sp. Nsp58	E. Spieck (unpublished)	Masonry, Germany
Nitrosospira sp. Nsp62	E. Spieck (unpublished)	Masonry, Germany
Nitrosospira sp. Nsp65	E. Spieck (unpublished)	Masonry, Germany
Nitrosospira tenuis Nv1^T	Koops & Harms (1985)	Soil, Hawaii
Nitrosospira sp. Nv6	Koops & Harms (1985)	Soil, New Guinea
Nitrosospira sp. NL5	Koops & Harms (1985)	Wastewater treatment plant, Saudi Arabia
Nitrosospira multiformis NL13^T	Koops & Harms (1985)	Soil, India
Nitrosospira sp. L115	Utåker *et al.* (1995)	Peat bog, Finland
Nitrosospira sp. III7	Utåker & Nes (1998)	Spruce forest, Norway
Nitrosospira sp. Ka3	Aakra *et al.* (1999b)	Soil, Norway

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positions were considered in the calculations. For a more detailed description of the phylogeny inference methods applied, see Purkhold *et al.* (2000).

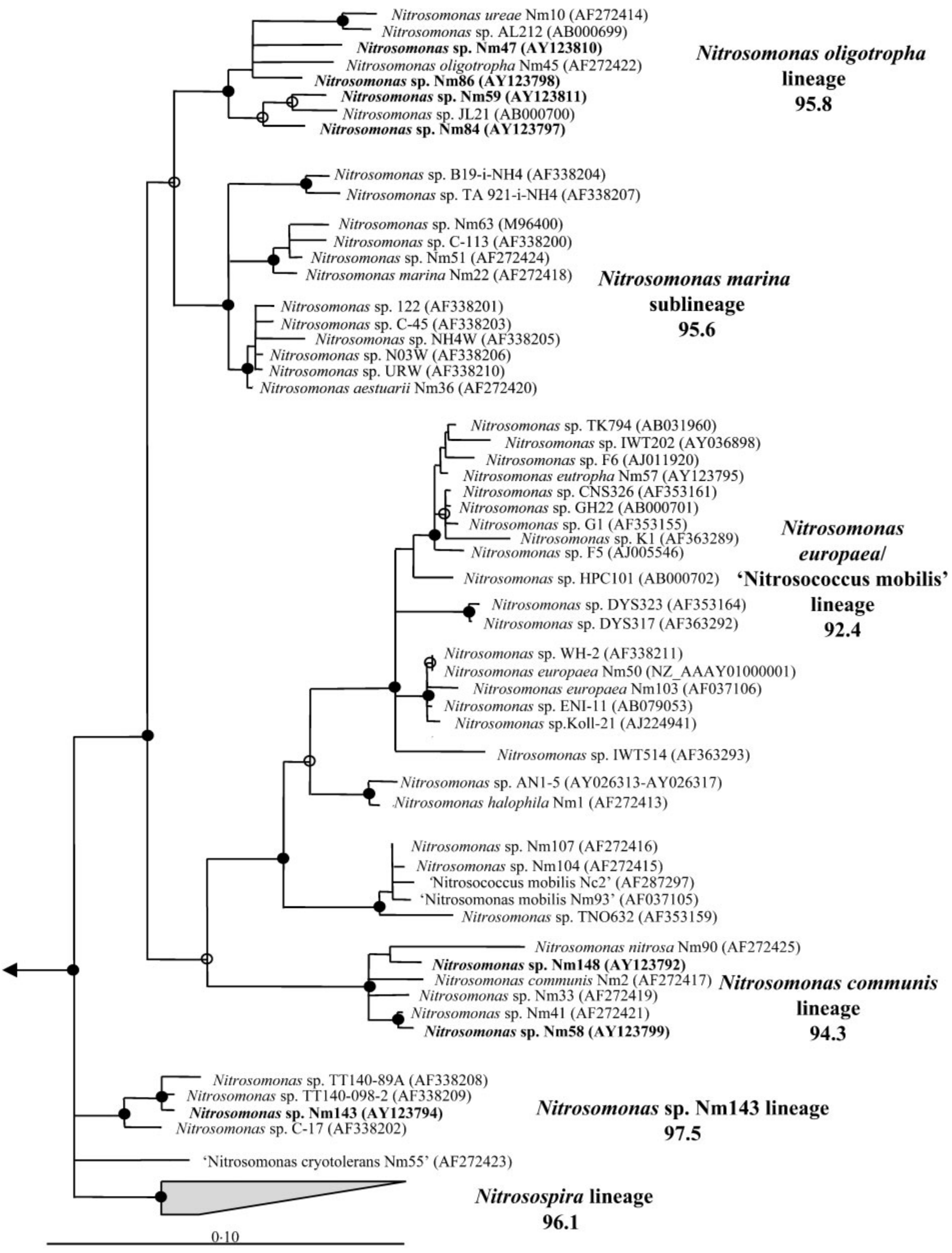
RESULTS AND DISCUSSION

AOB phylogeny inferred from 16S rRNA

To establish an encompassing high-quality 16S rRNA gene database for AOB, we resequenced the respective genes of several AOB isolates for which only incomplete sequences were available. In detail, 16S rRNA gene sequences (nt 1496–1498) were completed for *Nitrosomonas* spp. Nm58, Nm84 and Nm86, which represent isolates from the River Elbe (Stehr *et al.*, 1995a). For these strains, only very short 16S rRNA gene sequences (nt 186–281) have been published previously (Stehr *et al.*, 1995a). In addition, ambiguities and errors in the 16S rRNA gene sequences of *Nitrosospira* spp. Nsp1, Nsp2, Nsp12, Nsp17, Nv6 (all Aakra *et al.*, 2001b), Ka3 (Aakra *et al.*, 1999b), III7 (Utåker & Nes, 1998) and L115 (Utåker *et al.*, 1995) and *Nitrosospira briensis* Nsp10 (Aakra *et al.*, 2001b), *Nitrosomonas eutropha* Nm57 (Aakra *et al.*, 2001b; Head *et al.*, 1993), 'Nitrosovibrio tenuis Nv1' (Head *et al.*, 1993) and *Nitrosolobus multiformis* Nl13 (Teske *et al.*, 1994) were corrected and the sequences were extended by 23–290 bp to almost full-length (nt 1497–1498).

Furthermore, we determined almost full-length 16S rRNA gene sequences (nt 1494–1501) for the following 12 AOB isolates, which were not characterized at this level previously: *Nitrosomonas* spp. Nm47, Nm59, Nm143 and Nm148 and *Nitrosospira* spp. Nsp5, Nsp40, Nsp41, Nsp57, Nsp58, Nsp62, Nsp65 and NL5.

As expected, all 16S rRNA gene sequences determined showed highest similarities (96·7–100 %) to sequences of AOB belonging to the class '*Betaproteobacteria*' (available as supplementary data in IJSEM Online). Phylogenetic inference based on 16S rRNA gene sequences of AOB included distance-matrix, maximum-parsimony and maximum-likelihood methods and only considered sequences of more than 1000 nucleotides in length. All AOB analysed formed a monophyletic group within the class '*Betaproteobacteria*'. Within this group, 'Nitrosomonas cryotolerans' forms an independent lineage. In addition, five stable subgroupings (lineages *Nitrosomonas oligotropha*, *Nitrosomonas marina*, *Nitrosomonas europaea*/'Nitrosococcus mobilis', *Nitrosomonas communis* and *Nitrosospira*; Stephen *et al.*,



1996; Pommerening-Röser *et al.*, 1996; Purkhold *et al.*, 2000), as well as a lineage not recognized previously (including *Nitrosomonas* sp. Nm143) were recovered. All groupings are highly supported by parsimony bootstrap analysis (above 90 %) and were found independent from the treeing method applied (Fig. 1). The phylogenetic affiliation of the 12 AOB isolates newly sequenced is summarized below.

Phylogenetic relationship of the newly analysed nitrosomonads. *Nitrosomonas* isolates Nm47, Nm59, Nm84 and Nm86 are related most closely to organisms within the *Nitrosomonas oligotropha* lineage (97·0–97·5 %). As have many other members of this group, these isolates have been isolated from either freshwater or wastewater habitats and are characterized by remarkably low affinity constants for ammonia (Koops & Pommerening-Röser, 2001; Pommerening-Röser *et al.*, 1996; Stehr *et al.*, 1995a; Suwa *et al.*, 1994). Moreover, *Nitrosomonas* sp. Nm84 has been shown to produce significant amounts of exopolymeric substances, especially under conditions of limited ammonia (Stehr *et al.*, 1995b); this property has also been observed for other members of this lineage (H.-P. Koops, unpublished results).

Nitrosomonas spp. Nm58 and Nm148 can both be assigned unambiguously to the *Nitrosomonas communis* lineage (maximum sequence similarities 99·6 and 98·3 %, respectively; Fig. 1). *Nitrosomonas* sp. Nm148 has been isolated from a hot spring and is a strain of the species *Nitrosomonas nitrosa* (82 % DNA–DNA homology), which has been obtained from activated sludge of a wastewater treatment plant connected to chemical processing facilities (Koops *et al.*, 1991). The 16S rRNA gene sequence of *Nitrosomonas* sp. Nm58 is almost identical to the sequence of the soil isolate *Nitrosomonas* sp. Nm41 (99·6 %). The close relationship between both isolates is also reflected by their high DNA–DNA homology (71 %). In contrast to the *Nitrosomonas oligotropha* lineage, the *Nitrosomonas communis* lineage exhibits a high heterogeneity considering the ecophysiological traits of its members (Koops & Pommerening-Röser, 2001; Pommerening-Röser *et al.*, 1996).

The only 16S rRNA gene sequence obtained in this study that was not related directly to a published sequence from an AOB isolate was extracted from the estuarine isolate *Nitrosomonas* sp. Nm143. Considering only described species, the 16S rRNA gene of this organism shows highest sequence similarity to 'Nitrosomonas cryotolerans' (96·7 %). Together with the marine strains C-17, TT140-098-2 and TT140-89A, isolated from sediment samples at the Galapagos Islands and the Washington coast (GenBank accession nos AF338202, AF338209 and AF338208; maximum sequence similarity 99·0 %; Ward, 1982; Ward & Carlucci, 1985), *Nitrosomonas* sp. Nm143 forms a novel lineage within the betaproteobacterial AOB. This lineage is recovered with all treeing methods and is highly supported by bootstrapping. It comprises not only sequences of isolated strains but also harbours 17 16S rRNA gene sequences directly retrieved from different marine habitats (accession nos U09545–U09547, Z69090, AJ132050, AJ132056, AY114346, AY114347, AF489686–AF489689, Z69127, Z69134, Z69136, Z69141 and Z69143; de Bie *et al.*, 2001; McCaig *et al.*, 1994; Nicolaisen & Ramsing, 2002; Stephen *et al.*, 1996; Freitag & Prosser, 2003). In accordance with the current classification schemes (Pommerening-Röser *et al.*, 1996; Purkhold *et al.*, 2000; Stephen *et al.*, 1996), we propose to designate the new lineage as *Nitrosomonas* sp. Nm143 lineage. All isolates and sequences within this group originate from a total of eight distinct estuarine or marine habitats. Within these environments, members of the *Nitrosomonas* sp. Nm143 lineage seem to be distributed widely, since the sampling sites range from coastal surface water (McCaig *et al.*, 1994) to polluted (Stephen *et al.*, 1996) or even anoxic sediments (Freitag & Prosser, 2003). Nitrogen load and oxygen concentration at the various sampling sites differ significantly [polluted and non-polluted fish farm sediments (Stephen *et al.*, 1996), an eutrophic estuary (Nicolaisen & Ramsing, 2002) and estuarine sampling sites with ammonium concentrations below 15 mM (de Bie *et al.*, 2001) as well as anoxic sediments (Freitag & Prosser, 2003) and estuarine sites with oxygen saturation levels around 40 % (de Bie *et al.*, 2001)]. A common feature among the sites investigated are salinity values above 10 p.p.t. Interestingly, however, members of this lineage were not yet detected in the open sea (Bano & Hollibaugh, 2000; Phillips *et al.*, 1999; Hollibaugh *et al.*, 2002).

Phylogenetic relationship among the newly analysed nitrosospiras. As expected, *Nitrosospira* isolates NL5, Nsp5, Nsp40, Nsp41, Nsp57, Nsp58, Nsp62 and Nsp65 show the highest 16S rRNA gene similarities to sequences within the *Nitrosospira* lineage (98·6–100 %). *Nitrosospira*

Fig. 1. 16S rRNA-based phylogenetic tree of the nitrosomonads. The tree includes all isolates for which 16S rRNA gene sequences longer than 1000 nucleotides are available. Species whose sequences have been determined in this study are depicted in bold. Maximum-likelihood, maximum-parsimony and neighbour-joining trees were calculated and merged. Multifurcations connect branches for which a relative order cannot be determined unambiguously by applying different treeing methods. Filled and empty circles indicate parsimony bootstrap values (100 resamplings) above 90 and 70 %, respectively. For each lineage, the minimum 16S rRNA sequence similarity between two of its members is depicted. Sequences included in the analysis were published by Aakra *et al.* (1999a, b), Head *et al.* (1993), Juretschko *et al.* (1998), Purkhold *et al.* (2000), Sorokin *et al.* (1998), Suwa *et al.* (1997) and Yamagata *et al.* (1999). Sequences of strains CNS326, G1, K1, IWT202, TK794, WH-2, Koll-21, DYS317, DYS323, IWT514, TNO632, marine bacteria C-45, NH4W, 122, URW, NO3W, C-113, TT140-098-2, TT140-98A and estuarine bacteria TA 921-i-NH4, B19-i-NH4, C-17 are unpublished but are available at GenBank. Scale bar represents 10 % estimated sequence divergence.

isolates Nsp57 and Nsp58, which, according to DNA–DNA hybridization data, are members of the same species (63 % homology; note that AOB strains having more than 60 % DNA–DNA homology are considered as members of the same species; Koops *et al.*, 2003) group together but can be assigned neither to one of the clusters within the cultured nitrosospiras nor to the *Nitrosospira* cluster 1, which is composed entirely of environmentally retrieved sequences (Stephen *et al.*, 1996). Similarly *Nitrosospira* sp. Nsp65 forms an independent branch in the *Nitrosospira* lineage and currently represents the deepest branch within this evolutionary lineage. In contrast, isolate Nsp5 groups with cluster 0 and isolates Nsp 62, Nsp 41, Nsp 40 and NL5 are related most closely to members of the *Nitrosospira* cluster 3, which contains the three described species of this genus (Fig. 2). Although three of the 12 *Nitrosospira*-related isolates investigated cannot be assigned to previously suggested clusters of this lineage (Purkhold *et al.*, 2000; Stephen *et al.*, 1996), we refrain from proposing two novel clusters for these AOB because it has been noted that subdivision of nitrosospiras is not well supported by bootstrap analysis (Fig. 2; Purkhold *et al.*, 2000). The failure to recover stable clusters within the nitrosospiras reflects that 16S rRNA sequence similarities within the entire *Nitrosospira* lineage are higher ($>96{\cdot}1$ %) than those found within each of the *Nitrosomonas* lineages described. Furthermore, according to DNA–DNA hybridization data of available strains, *Nitrosospira* clusters 0 and 2 each currently encompass only strains from a single species. Within cluster 0, *Nitrosospira* spp. III2, 40KI, Nsp12 and Nsp5 possess DNA–DNA homology values with each other above 67 %. Within cluster 2, *Nitrosospira* spp. III7 and B6 share 76 % DNA–DNA homology. These values indicate that for both groupings of strains, the proposal of additional taxonomic units (clusters 0 and 2) is not justified at this time.

AOB phylogeny inferred from *amoA*

During the past few years, the gene encoding the active site subunit of *amoA* has been exploited increasingly as a marker molecule for AOB diversity research in natural and engineered systems (Baribeau *et al.*, 2000; Gieseke *et al.*, 2001; Horz *et al.*, 2000; Rotthauwe *et al.*, 1997). Initially, *amoA* gene fragment sequences published previously of *Nitrosospira* spp. Nsp1, Nsp2, Nsp12, Nsp17, Nv6, Ka3, III7 and L115, *Nitrosospira briensis* Nsp10 and 'Nitrosovibrio tenuis Nv1' (Aakra *et al.*, 2001a) were extended by 39 bp each to 453 bp, which represents the complete fragment obtained after PCR amplification using the modified (Stephen *et al.*, 1999) primer set of Rotthauwe *et al.* (1997). In addition, 453 bp long *amoA* sequences were determined for the 12 novel AOB isolates *Nitrosomonas* spp. Nm47, Nm59, Nm143 and Nm148 and *Nitrosospira* spp. Nsp5, Nsp40, Nsp41, Nsp57, Nsp58, Nsp62, NL5 and Nsp65. In addition, the *amoA* gene fragment sequences of the River Elbe isolates, *Nitrosomonas* spp. Nm58, Nm84 and Nm86, were determined.

All *amoA*/AmoA sequences determined showed the highest similarity (83·2–99·3 and 90·7–100 %, respectively) to sequences of AOB belonging to the class '*Betaproteobacteria*' (available as supplementary data in IJSEM Online). Phylogenetic trees for *amoA*/AmoA were calculated from the nucleotide and amino acid datasets by distance-matrix, maximum-parsimony and maximum-likelihood methods. In general, topologies of *amoA*/AmoA- and 16S rRNA-based trees were very similar (Figs 1 and 3). The monophyly of the *Nitrosospira* lineage, the *Nitrosomonas marina* lineage and the *Nitrosomonas europaea*/'Nitrosococcus mobilis' lineage was recovered by all methods, although the bootstrap support for these lineages was considerably lower than that found for 16S rRNA gene trees. In contrast to 16S rRNA trees, the *Nitrosomonas oligotropha* lineage and the *Nitrosomonas communis* lineage are not always retrieved in *amoA*/AmoA trees as monophyletic assemblages (Purkhold *et al.*, 2000).

For all AOB strains for which the *amoA*/AmoA sequence was newly determined in this study, consistent affiliations were found in the 16S rRNA gene- and *amoA*/AmoA-based trees (Figs 1 and 3). However, the *amoA*/AmoA sequences of the nitrosospiras analysed offer insufficient resolution for inferring details on the phylogenetic substructure of this lineage. The clusters proposed previously within the nitrosospiras are not always monophyletic in *amoA*/AmoA trees (dependent on the treeing method used) and the bootstrap support of the clusters is very low (data not shown). As expected, the *amoA*/AmoA sequence of *Nitrosomonas* sp. Nm143, the organism representing the novel lineage in the 16S rRNA AOB tree, is not closely related to *amoA*/AmoA sequences of cultured AOB in the database. However, some, but not all, treeing methods suggest a weak affiliation of the *amoA*/AmoA sequence of *Nitrosomonas* sp. Nm143 with members of the *Nitrosomonas marina* and/or *Nitrosomonas oligotropha* lineages.

With the extending dataset and an increasing number of closely related *amoA*/AmoA sequences, the limitation of the *amoA*/AmoA approach as applied now becomes more apparent. Although using the *amoA* approach, AOB pure cultures or AOB in environmental samples can be assigned rapidly to some phylogenetic subgroups within this guild (see above), the *amoA*/AmoA fragment analysed does provide less resolution compared to the 16S rRNA, since it is relatively short (453 nt and 151 aa positions, respectively) and highly conserved (224/93 positions have an identical nucleotide/amino acid in at least 98 % of the betaproteobacterial AOB). This limitation might be solved in future studies by application of primers that allow the amplification of a longer *amoA* fragment (Norton *et al.*, 2002).

Inconsistencies between determined sequences and published database entries

To improve the respective databases, we resequenced in this study several 16S rRNA and *amoA* sequences of defined isolates. Comparison of the newly determined sequences

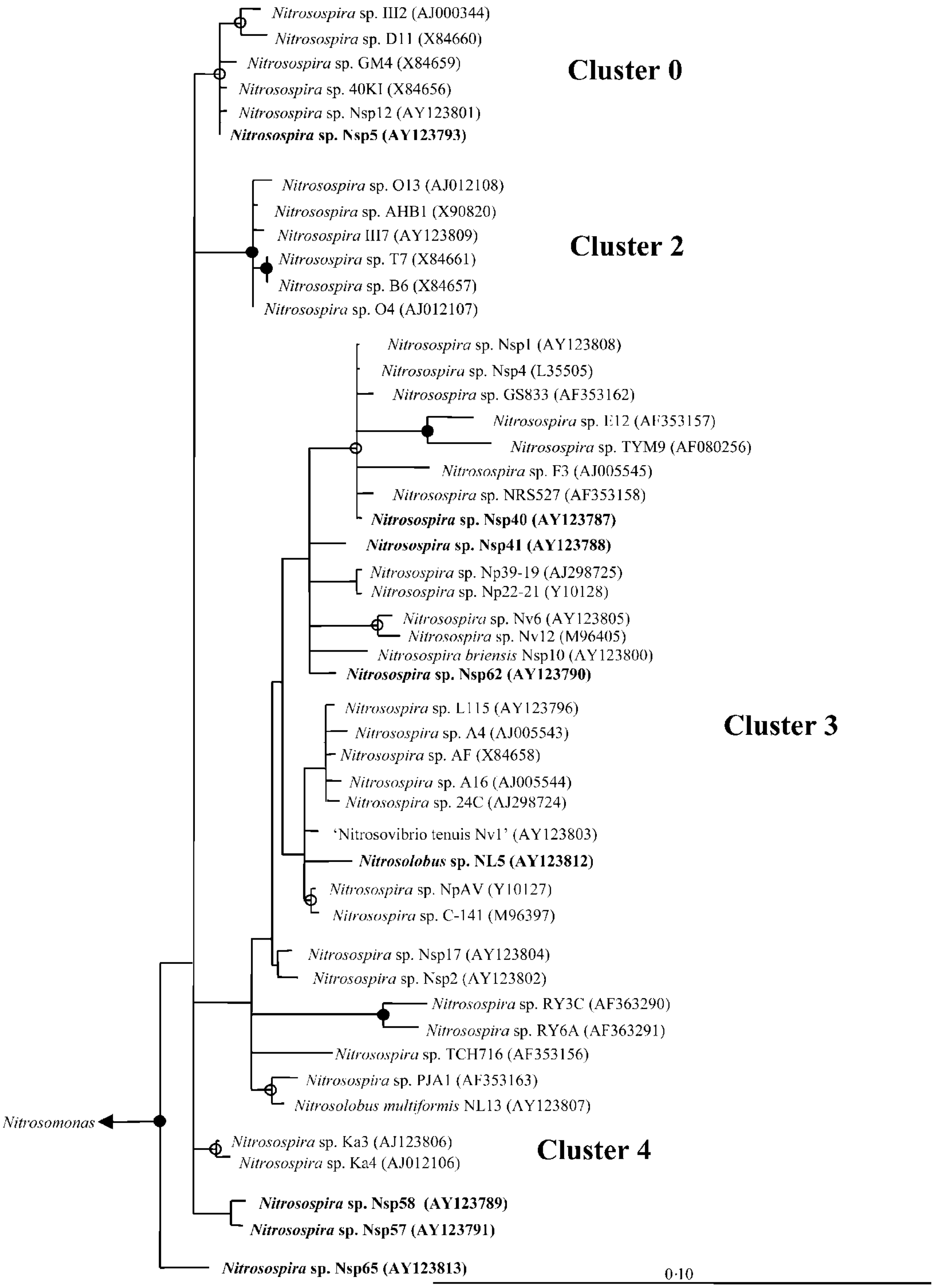


Fig. 2. 16S rRNA-based phylogenetic tree of the highly related genera *Nitrosospira*, *Nitrosolobus* and 'Nitrosovibrio' forming the *Nitrosospira* lineage (Head *et al.*, 1993). The tree includes all isolates for which 16S rRNA gene sequences longer than 1000 nucleotides are available. Species whose sequences have been determined in this study are depicted in bold. Maximum-likelihood, maximum-parsimony and neighbour-joining trees were calculated and merged. Multifurcations connect branches for which a relative order cannot be determined unambiguously by applying different treeing methods. Filled and empty circles indicate parsimony bootstrap values (100 resamplings) above 90 and 70 %, respectively. Sequences included in the analysis were published by Aakra *et al.* (1999a, b, 2001b), Head *et al.* (1993), Teske *et al.* (1994), Tokuyama *et al.* (1997) and Utåker *et al.* (1995). Sequences of strains GS833, E12, NRS527, NpAV, RY6A, RY3C, TCH716 and PJA1 are unpublished but are available at GenBank. Scale bar represents 10 % estimated sequence divergence.

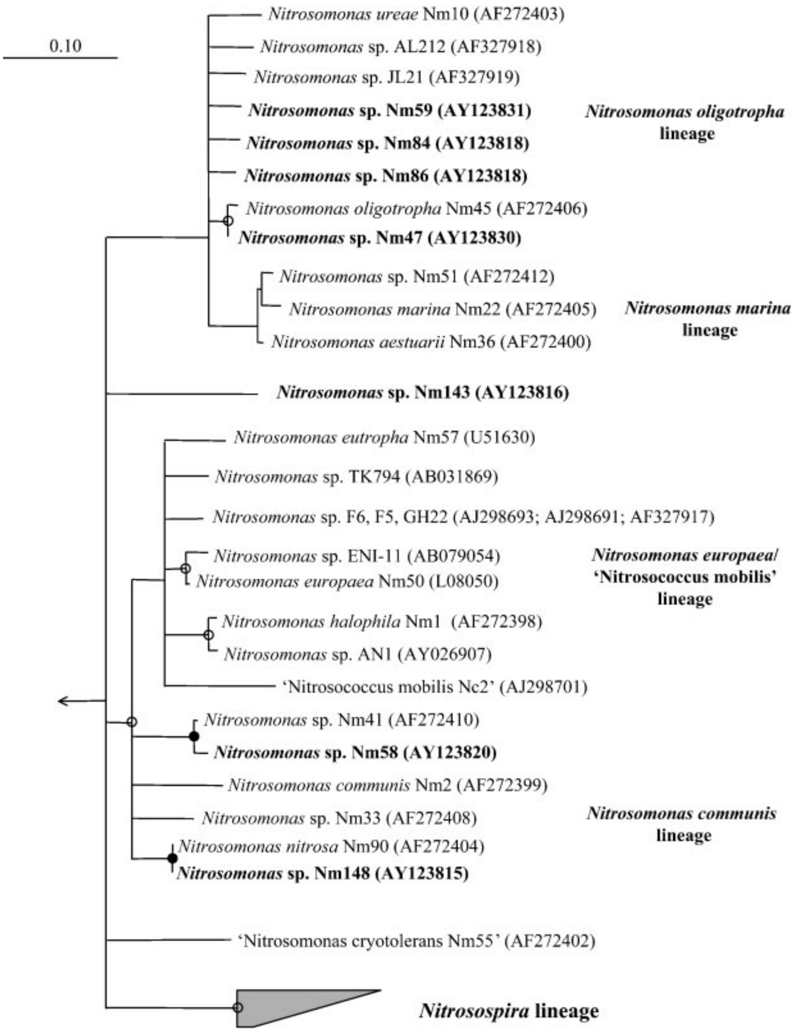


Fig. 3. AmoA-based phylogenetic tree of the betaproteobacterial AOB. Species whose sequences have been determined in this study are depicted in bold. The 453 bp gene fragment obtainable with the *amoA* PCR primers used most commonly (Rotthauwe *et al.*, 1997) was used for phylogeny inference. AmoA sequences shorter than 414 nucleotides were excluded from the analysis. Protein maximum-likelihood, protein maximum-parsimony, neighbour-joining and FITCH trees were calculated and merged. Multifurcations connect branches for which a relative order cannot be determined unambiguously by applying different treeing methods. Filled and empty circles indicate parsimony bootstrap values (100 resamplings) above 90 and 70 %, respectively. Sequences included in the analysis were published by Aakra *et al.* (2001a), Casciotti & Ward (2001), Holmes *et al.* (1995), McTavish *et al.* (1993), Norton *et al.* (2002), Purkhold *et al.* (2000), Rotthauwe *et al.* (1995), Sorokin *et al.* (2001), Suwa *et al.* (1997) and Yamagata *et al.* (1999). Sequences of *Nitrosospira* sp. C-57 and *Nitrosomonas* sp. TK794 are unpublished but are available at GenBank. Scale bar represents 10 % estimated sequence divergence.

with those sequences published previously by others revealed several inconsistencies that could not be explained by simple sequencing errors.

Firstly, differences in the *amoA*/AmoA sequences (97·6 and 99·2 %, respectively) of *Nitrosospira* sp. III7 determined in this study and those published by Aakra *et al.* (2001a) were detected. However, both sequences show the same phylogeny and, therefore, probably represent two different gene copies. The existence of more than one *amoA* gene copy is common among betaproteobacterial AOB and up to four copies have been reported to occur within the genomes of some nitrosospiras (Bock & Wagner, 2001).

Secondly, we came across published sequences that were obviously retrieved from a contaminant and have not been extracted from the indicated AOB. The 16S rRNA gene sequence of '*Nitrosospira* sp. Nv1' published by Aakra *et al.* (2001b) differs significantly (sequence similarity 98·6 %) from the sequences determined in this study (accession no. AY123803) and by Head *et al.* (1993) (accession no. M96404) and which are almost identical (99·9 %) to each other. It seems likely that the latter two sequences are correct also because the close association of *Nitrosospira* sp. Nv1 with *Nitrosospira* sp. Nv12 (accession no. M96405, 99·8 %; Head *et al.*, 1993) is not supported by DNA–DNA hybridization data, which demonstrate that the two organisms belong to different species (Pommerening-Röser, 1993). Furthermore, the *amoA* fragment of *Nitrosospira* sp. L115 (Aakra *et al.*, 2001a) shows significant sequence differences to the respective sequence determined in this study (nucleic acid, 88·9 %; amino acid, 94·6 %). We claim our sequence to be correct since (in contrast to the sequence of Aakra and co-workers) the results of its phylogenetic analysis are in accordance with the respective 16S rRNA phylogeny (Fig. 2).

Conclusion

This study extended significantly the current 16S rRNA and *amoA* databases for AOB. For several AOB isolates, previously published sequences of both marker molecules were improved in quality and length. Furthermore, gene sequences of both macromolecules were determined for 12 novel AOB isolates. Based on these data, a thorough phylogenetic analysis was performed, which led to the description of a new 16S rRNA gene lineage within the nitrosomonads. This lineage also contains 17 previously unassigned environmental clones, demonstrating that at least one of the new AOB lineages discovered during the past few years by molecular diversity surveys harbours AOB species that can be cultured by traditional techniques.

ACKNOWLEDGEMENTS

This study was supported by a grant of the DFG to M. W. (WA1558/1) and a grant from the bmb+f to M. W. (01 LC 0021 subproject 2, in the framework of the BIOLOG programme). The excellent technical assistance of Sibylle Schadhauser is acknowledged. We kindly thank Thomas Freitag for providing environmentally retrieved 16S rRNA gene sequences prior to publication.

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