Detailed phylogeny of ammonia-oxidizing bacteria determined by rDNA sequences and DNA homology values

¹ Laboratory of Microbial Gene Technology, Department of Chemistry and Biotechnology, Agricultural University of Norway, PO Box 5051, N-1432 Ås, Norway

² Institut für Allgemeine Botanik, Abteilung Mikrobiologie, Universität Hamburg, Ohnhorststrasse 18, D-22609 Hamburg, Germany

Ågot Aakra,¹ Janne B. Utåker,¹ Andreas Pommerening-Röser,² Hans-Peter Koops² and Ingolf F. Nes¹

Author for correspondence: Ågot Aakra. Tel: +47 6494 9456. Fax: +47 6494 1465. e-mail: agot.aakra@ikb.nlh.no

A comparison of the phylogeny of 38 isolates of chemolithoautotrophic ammonia-oxidizing bacteria (AOB) based on 16S rRNA gene sequences, 16S–23S rDNA intergenic spacer region (ISR) sequences and species affiliations based on DNA homology values was performed. The organisms studied all belong to the β -subclass of the *Proteobacteria* and included representatives of *Nitrosomonas*, *Nitrosococcus* and *Nitrospira*. The similarity values of the 16S rDNA sequences were high, particularly within the *Nitrospira* genus, and based on these sequences it is difficult to determine the phylogenetic position of some AOB. As an alternative and supplement to 16S rRNA gene sequencing, the ISR was sequenced and analysed phylogenetically. Due to considerably lower similarity values, the ISR-based phylogeny gives a better resolution than the phylogeny based on the functional 16S rRNA gene. Since the ISR-based phylogeny of AOB is highly consistent with the 16S rDNA based phylogeny, ISR sequencing appears as a suitable tool for resolving the detailed phylogeny of AOB. The phylogenetic position of two isolates of the former genus '*Nitrosolobus*' (now included in the *Nitrospira* genus) is not clear. These organisms are close relatives of the former *Nitrospira* spp. and '*Nitrosovibrio*' spp. (now *Nitrospira*), but based on their marginal positions in the phylogenetic trees, DNA–DNA hybridization data and phenotypic characteristics, it is suggested that '*Nitrosolobus*' should be a separate genus. DNA homology determination of 11 *Nitrospira* isolates revealed two new species of *Nitrospira*. The phylogeny of AOB reflected in the trees based on the rDNA sequences is consistent with the species affiliations of AOB by DNA homology values. This observation will probably be important for the interpretation of results from studies of natural diversity of AOB.

Keywords: chemolithoautotrophic ammonia-oxidizing bacteria, phylogeny, species determination

INTRODUCTION

With the exception of two marine species, all known chemolithoautotrophic ammonia-oxidizing bacteria (AOB) constitute a monophyletic group within the β -

subclass of the *Proteobacteria*. Over the last few years, the phylogeny of AOB based on 16S rDNA sequences has been studied, resulting in reclassification. Based on 16S rDNA sequence similarities, the three previously recognized genera '*Nitrosolobus*', *Nitrospira* and '*Nitrosovibrio*' were combined into one genus, *Nitrospira* (Head *et al.*, 1993, 1995). 16S rRNA gene sequence information has made it possible to design specific oligonucleotides for the direct monitoring of various subgroups of AOB in the environment (McCaig *et al.*, 1994; Utåker & Nes, 1998). The use of these specific oligonucleotides along with activity

Abbreviations: AOB, chemolithoautotrophic ammonia-oxidizing bacteria; ISR, 16S–23S rDNA intergenic spacer region.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA and ISR sequences determined in this study are AJ298724, AJ298725, AJ298728–AJ298730, AJ298732–AJ298739, AJ298741–AJ298747 (16S rDNA) and AJ298749–AJ298774 (ISR).

measurements have concluded that the diversity of AOB in the environment is high and that species other than *Nitrosomonas* spp. contribute significantly to the oxidation of ammonia to nitrite in nature (Aakra *et al.*, 2000; Jiang & Bakken, 1999; Kowalchuk *et al.*, 1997; McCaig *et al.*, 1994; Stephen *et al.*, 1996). However, the limited number of 16S rDNA sequences from pure cultures available biases these results, and the specificity of the oligonucleotides used as PCR primers or hybridization probes has been questioned (Aakra *et al.*, 2000; Utåker & Nes, 1998).

The similarity values between 16S rDNA sequences from some AOB are so high that the few nucleotides distinguishing them may be ascribed to sequencing ambiguities rather than true sequence differences (Utåker *et al.*, 1995). Hence, alternative techniques to 16S rRNA gene sequencing are needed to clarify the phylogeny of many β -proteobacterial AOB. Analysis of the 16S–23S rDNA intergenic spacer region (ISR) is one such technique, which can be applied as a supplement to 16S rRNA gene sequencing. ISRs evolve much faster than 16S rRNA genes, probably because of less functional restriction, and can, thus, be used to trace more recent evolutionary events (species and strain level) (Barry *et al.*, 1991; Garcia-Martinez *et al.*, 1999; Gürtler & Stanisich, 1996). Several studies have demonstrated the relevance of ISR sequence analysis for phylogenetic purposes, most of which have been performed with pure cultures of micro-organisms (Barry *et al.*, 1991; Garcia-Martinez *et al.*, 1999; Gürtler & Stanisich, 1996; Jensen *et al.*, 1993). Compared to 16S rRNA gene analysis, the use of ISR analysis has technical advantages: ISRs are usually shorter than the 16S rRNA gene, which makes sequencing less laborious. In some organisms differently sized ISRs may be easily separated, by standard agarose gel electrophoresis; cloning might not be necessary. More ISR sequences are needed, however, to increase the practical value of environmental studies based on ISR analysis. At present, the number of ISR sequences available in the international sequence databases is relatively low, which limits the use of ISR sequences in studies of microbial diversity. Moreover, the sequence variation between ISRs from different *rrn* operons within a bacterium may be as large as the sequence variation between ISRs from different organisms (Jensen *et al.*, 1993; Nagpal *et al.*, 1998). Consequently, ISR analysis of natural populations of bacteria with a single or few *rrn* operons per cell will be more applicable. The potential of exploiting the ISR in studies of natural samples has been shown (Acinas *et al.*, 1999; Borneman & Triplett, 1997; Robleto *et al.*, 1998). The results from these studies show that ISR analysis is a promising tool for characterization of natural microbial populations. In a previous study of 12 AOB isolates, it was shown that these organisms have only one *rrn* operon per genome and analysis of ISR should therefore be a suitable and useful tool for studies of AOB (Aakra *et al.*, 1999a).

Species determination of more cultured AOB (*Nitro-*

sospira spp. in particular) is needed to determine whether there is good correlation between phylogeny based on rDNA and species affiliations. If DNA sequence information from environmental samples can be used to infer the species identity of the organisms found in the actual environment, the interpretation can be simplified and give an improved resolution of the complexity of natural microbial populations. The basis for species determination of bacteria is DNA–DNA hybridization data. The DNA of strains within one bacterial species should reveal 50–70% DNA–DNA reassociation and phenotypic criteria should agree with the DNA homology values. In fact, phenotypic data for strains are decisive of whether a species should be delineated at 60 or 80% similarity level (Stackebrandt & Goebel, 1994). 16S rDNA sequence data cannot be used to determine species identity, but 16S rDNA sequence similarity values below 97% indicate that the organisms belong to different species (Stackebrandt & Goebel, 1994).

In this paper, the evolution of 38 AOB isolates has been studied by comparison of 16S rRNA gene-based phylogeny with ISR sequence-based phylogeny and species identities based on DNA homology values. On the basis of this information, the suitability and relevance of 16S rDNA and ISR for studies of AOB diversity has been evaluated.

METHODS

Bacteria and growth conditions. The AOB were grown in the dark at room temperature (22–24 °C). The origin and abbreviations for the isolates studied are given in Table 1. Terrestrial isolates (*Nitrosospira* and some *Nitrosomonas* isolates, see Table 1) were grown in a liquid ammonium medium as described by Jiang & Bakken (1999). Isolates from brackish water and marine habitats (*Nitrosococcus* and some *Nitrosomonas* isolates) were grown in the media described by Koops *et al.* (1991).

Cell lysis and preparation of PCR-ready DNA. Preparation of PCR-ready DNA was performed using Dynabeads DNA Direct system I (Dyna) as recommended by the manufacturer.

Amplification of 16S rDNA and the 16S–23S rDNA ISR. PCRs (50 or 100 µl) were run with *Taq* polymerase (1·25 or 2·5 U) (Advanced Biotechnologies), 10 mM Tris/HCl (pH 8·3), 50 mM KCl, 1·5 mM MgCl₂, 1 or 2% (v/v) formamide, 200 µM dNTP, 1 µM each primer (Gibco-BRL) and 1–2 µl (25–50 ng) genomic DNA, prepared as described above. All PCRs were run in a PTC-100 (Programmable Thermal Controller; MJ Research). All primers used are listed in Table 2. 16S rDNA was amplified using the primers 1F and 5R (Table 2) during 35 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min 30 s. The ISR was amplified using the primers 7F and 13R (Table 2) during 35 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min. All PCRs were preceded by a 3 min hot start at 97 °C and ended by a 10 min incubation at 72 °C. Prior to sequencing, PCR products were purified using silica gel columns (QIAquick PCR Purification Kit; Qiagen).

DNA sequencing. Cycle sequencing was performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism 377 DNA sequencer, as

Table 1. Abbreviations and origin of the organisms studied

Isolate (abbreviation)	Origin/strain no.	Reference
<i>Nitrosomonas communis</i> Nm2 ^T (Nm2)	Soil, Corfu	Koops <i>et al.</i> (1991)
<i>Nitrosomonas ureae</i> Nm10 ^T (Nm10)	Soil, Sardinia	Koops <i>et al.</i> (1991)
<i>Nitrosomonas marina</i> Nm22 ^T (Nm22)	Sea water, South Pacific	Koops <i>et al.</i> (1991)
<i>Nitrosomonas</i> sp. Nm33 (Nm33)	Soil, Japan	Koops <i>et al.</i> (1991)
<i>Nitrosomonas aestuarii</i> Nm36 ^T (Nm36)	Sea water, North Sea	Koops <i>et al.</i> (1991)
<i>Nitrosomonas</i> sp. Nm41 (Nm41)	Soil, St Petersburg	Koops <i>et al.</i> (1991)
<i>Nitrosomonas oligotropha</i> Nm45 (Nm45)	Soil, Hamburg	Koops <i>et al.</i> (1991)
<i>Nitrosomonas europaea</i> Nm50 ^T (Nm50)	Soil, USA	Type strain NCIMB 11850 ^T
<i>Nitrosomonas</i> sp. Nm51 (Nm51)	Sea water, Gulf of Maine	Koops <i>et al.</i> (1991)
<i>Nitrosomonas cryotolerans</i> Nm55 ^T (Nm55)	Sea water, Alaska/ATCC 49181	Jones <i>et al.</i> (1988)
<i>Nitrosomonas eutropha</i> Nm57 ^T (Nm57)	Municipal sewage, Chicago	Koops & Harms (1985)
<i>Nitrosomonas</i> sp. F5 (F5)	Animal house, Norway	Aakra <i>et al.</i> (1999a, b)
<i>Nitrosomonas</i> sp. F6 (F6)	Animal house, Norway	Aakra <i>et al.</i> (1999a, b)
<i>Nitrosospira multififormis</i> NI13 ^T (NI13)	Soil, Surinam/NCIMB 11849	Koops & Harms (1985)
<i>Nitrosospira</i> sp. NI20 (NI20)	Soil, Italy	H.-P. Koops (unpublished)
<i>Nitrosospira</i> sp. 24C (24C)	Agricultural soil, Minnesota	E. Schmidt (unpublished)
<i>Nitrosospira</i> sp. Nsp1 (Nsp1)	Soil, Sardinia	Koops & Harms (1985)
<i>Nitrosospira</i> sp. Nsp2 (Nsp2)	Soil, Hamburg	Koops & Harms (1985)
<i>Nitrosospira briensis</i> Nsp10 ^T (Nsp10)	Soil, Crete/ATCC 25071	Koops & Harms (1985)
<i>Nitrosospira</i> sp. Nsp12 (Nsp12)	Soil, Hamburg	Koops & Harms (1985)
<i>Nitrosospira</i> sp. Nsp17 (Nsp17)	Soil, Iceland	Koops & Harms (1985)
<i>Nitrosospira</i> sp. 39–19 (39–19)	Agricultural soil, Minnesota	E. Schmidt (unpublished)
<i>Nitrosospira</i> sp. 40KI (40KI)	Loam, garden soil, Norway	Utåker <i>et al.</i> (1995)
<i>Nitrosospira</i> sp. A16 (A16)	Agricultural soil, Norway	Aakra <i>et al.</i> (1999a, b)
<i>Nitrosospira</i> sp. A4 (A4)	Agricultural soil, Norway	Aakra <i>et al.</i> (1999a, b)
<i>Nitrosospira</i> sp. AF (AF)	Sandy loam, Zambia	Utåker <i>et al.</i> (1995)
<i>Nitrosospira</i> sp. B6 (B6)	Sewage treatment column, Norway	Utåker <i>et al.</i> (1995)
<i>Nitrosospira</i> sp. F3 (F3)	Animal house, Norway	Aakra <i>et al.</i> (1999a, b)
<i>Nitrosospira</i> sp. III2 (III2)	Spruce forest, Norway	Utåker & Nes (1998)
<i>Nitrosospira</i> sp. III7 (III7)	Spruce forest, Norway	Utåker & Nes (1998)
<i>Nitrosospira</i> sp. Ka3 (Ka3)	Lead-contaminated soil, Norway	Aakra <i>et al.</i> (1999a, b)
<i>Nitrosospira</i> sp. Ka4 (Ka4)	Lead-contaminated soil, Norway	Aakra <i>et al.</i> (1999a, b)
<i>Nitrosospira</i> sp. L115 (L115)	Peat bog, Finland	Utåker <i>et al.</i> (1995)
<i>Nitrosospira</i> sp. O13 (O13)	Agricultural soil, Norway	Aakra <i>et al.</i> (1999a, b)
<i>Nitrosospira</i> sp. O4 (O4)	Agricultural soil, Norway	Aakra <i>et al.</i> (1999a, b)
<i>Nitrosospira tenuis</i> Nv1 ^T (Nv1)	Soil, Hawaii	Koops & Harms (1985)
<i>Nitrosospira</i> sp. Nv6 (Nv6)	Soil, Goroko/Danlo Pas	Koops & Harms (1985)
<i>'Nitrosococcus mobilis'</i> Nc2 ^T (Nc2)	Sea water, North Sea	Koops & Harms (1985)

recommended by the supplier (Applied Biosystems). To sequence the 16S rRNA gene, the primers 1F, 11F, 15F, 16F, 9R, 3R, 4R and 5R (Table 2) were used, giving overlapping sequences, on both strands. For ISR sequencing the primers 7F, 5F, ISR-F, ISR-R and 13R (Table 2), also covering both strands, were applied.

Data analysis. The sequences obtained were analysed and edited using programs in the Genetics Computer Group (GCG) program package, version 8.1 (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, Madison, WI, USA). Alignments of sequences were performed in CLUSTAL W (<http://www2.ebi.ac.uk/clustalw/>) (Thompson *et al.*, 1994). Prior to construction of phylogenetic trees the alignments were carefully corrected manually and the 16S rRNA gene sequences were adjusted to equal lengths (approx. 1205 bp). The ISR sequences were stripped from the 3' end of the 16S rRNA gene and the 5' end of the 23S

rRNA gene. Phylogenetic analysis was performed by using different methods: distance matrix (neighbour-joining), maximum-parsimony and maximum-likelihood. The programs used for the phylogenetic analysis were DNADIST, NEIGHBOUR, DNAPARS, SEQBOOT, CONSENSE and DNAML in the PHYLIP program package, version 3.5 (Felsenstein, 1993). Bootstrap analyses of neighbour-joining and maximum-parsimony trees were performed with 100 replicates. Similarity values for the sequences were estimated using the program HOMOLOGIES in the EGCG program package (Program Manual for the EGCG Package, Version 8.1.0, March 1996, Hinxton Hall, Cambridge). The GCG, EGCG and PHYLIP programs were all run on a UNIX mainframe computer. Phylogenetic trees were visualized using TREEVIEW software, version 1.5 (Page, 1996) (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

DNA sequence accession numbers. The accession numbers are indicated in Fig. 1. Nearly full-length 16S rDNA

sequences of Nm2, Nm10, Nm55, Nm57 and Nc2 have been published previously (Head *et al.*, 1993; Pommerening-Röser *et al.*, 1996), but to resolve some of the ambiguities of these sequences, they have been sequenced again here. The following sequences were extracted from the nucleotide sequence databases: *Nitrosospira* sp. AF (16S rDNA and ISR), *Nitrosospira* sp. B6 (16S rDNA and ISR), *Nitrosospira* sp. 40KI (16S rDNA and ISR), *Nitrosospira* sp. L115 (16S rDNA and ISR), *Nitrosospira* sp. III2 (16S rDNA and ISR), *Nitrosospira* sp. III7 (16S rDNA and ISR), *Nitrosomonas europaea* Nm50 (16S rDNA and ISR), *Nitrosospira multiformis* N113 (16S rDNA and ISR), *Nitrosospira* sp. F3 (16S rDNA and ISR), *Nitrosomonas* sp. F5 (16S rDNA and ISR), *Nitrosospira* sp. A4 (16S rDNA and ISR), *Nitrosospira* sp. A16 (16S rDNA and ISR), *Nitrosospira* sp. Ka3 (16S rDNA), *Nitrosospira* sp. Ka4 (16S rDNA), *Nitrosospira* sp. O4 (16S rDNA), *Nitrosospira* sp. O13 (16S rDNA), *Nitrosomonas* sp. F6 (16S rDNA), *Nitrosomonas marina* Nm22 (16S rDNA).

DNA–DNA homology determination. DNA–DNA hybridizations were performed as described previously (Koops & Harms, 1985).

RESULTS AND DISCUSSION

Nearly full-length 16S rDNA sequences were obtained for the *Nitrosospira* isolates 24C, 39–19, N120, Nsp1, Nsp2, Nsp10, Nsp12, Nsp17, Nv1 and Nv6, for the *Nitrosomonas* isolates Nm2, Nm10, Nm33, Nm36, Nm41, Nm45, Nm51, Nm55 and Nm57, and for ‘*Nitrosococcus mobilis*’ Nc2 (Figs 1 and 2, Table 1). These new 16S rDNA sequences were aligned to previously published 16S rDNA sequences of AOB and all sequences were adjusted to equal length (approx. 1205 bp) before phylogenetic analysis. Furthermore, the ISR of the *Nitrosospira* isolates 24C, 39–19, N120, Nsp1, Nsp2, Nsp10, Nsp12, Nsp17, Nv1, Nv6, Ka3, Ka4, O4 and O13, the *Nitrosomonas* isolates F6, Nm2, Nm22, Nm10, Nm33, Nm36, Nm41, Nm45, Nm51, Nm55 and Nm57, and ‘*Nitrosococcus mobilis*’ Nc2 were sequenced and analysed. For phylogenetic analysis of the 16S rRNA gene and ISR sequences neighbour-joining, maximum-parsimony and maximum-likelihood analyses were performed. However, neighbour-joining appeared to be the most suitable method for phylogenetic analysis and comparison of these two datasets. Maximum-parsimony is probably a suitable method for phylogenetic analysis of the highly similar 16S rDNA sequences from AOB. For analysis of the more variable ISR sequences, however, parsimony analysis appears more problematic. Most likely, the rapid evolution of the ISR sequences may have led to saturation of several nucleotide sites. In a parsimony analysis such saturated sites may be misleading; they will be overprinted and lost. Nevertheless, both datasets were analysed by neighbour-joining and maximum-parsimony, with bootstrapping, to test the precision of the branching patterns, and by the maximum-likelihood method. The branching patterns of the trees obtained by the different methods were the same and only the neighbour-joining distance trees are shown

here (Figs 1 and 2). The topology of the trees in Fig. 1 and their corresponding majority rule consensus trees of the bootstrap analyses (Fig. 2) were nearly identical. In the phylogenetic trees shown (Figs 1 and 2) the organisms have been divided into groups (named NmA–C for *Nitrosomonas* spp. and NspA–D for *Nitrosospira* spp.) based upon their clustering patterns in the trees. Some of the groups contain representatives of more than one species, as defined by DNA homology values. However, isolates belonging to one species do all cluster within the same phylogenetic group, in which representatives of another species may accompany them or not.

Phylogeny of AOB

16S rDNA-based phylogeny: *Nitrosomonas*. Within the *Nitrosomonas* genus, the resolution of the phylogeny based on 16S rDNA sequences is relatively good, considering the overall high similarity values of these sequences among AOB. Most of the nodes are supported with high bootstrap values (> 50%; Fig. 2). The terrestrial *Nitrosomonas* isolates (F5, F6, Nm50, Nm57, Nm2, Nm33 and Nm41) can be distinguished from marine *Nitrosomonas* isolates (Nm22, Nm36, Nm51 and Nm55), although the node separating Nm2, Nm33 and Nm41 from Nm10, Nm22, Nm36, Nm45 and Nm51 (cluster NmB) is supported by only 48% of the bootstrap replicates. The Nm55 isolate is separated from the other *Nitrosomonas* isolates, being the *Nitrosomonas* isolate most closely related to the *Nitrosospira* group (bootstrap value 85%), based on 16S rDNA sequences. The 16S rDNA sequence similarities in the *Nitrosomonas* cluster vary from 90.9% (between F5 and Nm10) to 99.5% (between F6 and Nm57). The similarity values of the 16S rDNA sequences of Nm36, Nm22 and Nm51 (in cluster NmB) are higher than 97.0%, indicating that these organisms may be strains of the same species (Stackebrandt & Goebel, 1994). However, DNA homology values show that they all belong to different species (Koops *et al.*, 1991). Likewise, the 16S rDNA similarity value for Nm50 and Nm57 (cluster NmA) is 97.5%. For Nm41, Nm33 and Nm2 (cluster NmB) the similarity values vary between 97.1 and 98.3%, but the DNA homology values are so low that they can be assigned to three different species (Koops *et al.*, 1991; Koops & Harms, 1985). These data demonstrate that within the *Nitrosomonas* genus, nearly identical 16S rDNA sequences do not guarantee species identity, a phenomenon that has been described also for other organisms (Fox *et al.*, 1992). The DNA homologies between F5, F6, Nm50 and Nm57 have not been determined, since we were not able to obtain sufficient amounts of cell material of F5 and F6 to perform the DNA–DNA hybridization experiments. Hence, their species affiliations are still unknown.

16S rDNA-based phylogeny: *Nitrosospira*. Among the *Nitrosospira* isolates, the result of the phylogenetic analysis of the 16S rDNA sequences is more complex.

Table 2. Oligonucleotides used for amplification and sequencing of 16S rDNA and ISR

Primer	Sequence (5'–3')	Target	Position in target	Reference
1F	gagtttgatcctggctcag	16S rDNA	9–27*	Weisburg <i>et al.</i> (1991)
11F	tggcgaagcgcccccctgga	16S rDNA	50–70*	Edwards <i>et al.</i> (1989)
15F	acgggaggcagcagt	16S rDNA	343–357*	Stackebrandt & Charfreitag (1990)
16F	aactcaaatgaattgacggg	16S rDNA	907–926*	Lane <i>et al.</i> (1985)
7F	gccttgatcacaccgc	16S rDNA	1386–1401*	Modified from Lane <i>et al.</i> (1985)
5F	aagtcgtaacaaggttaacc	16S rDNA	1492–1510*	Stackebrandt & Charfreitag (1990)
ISR-F	tgataagcgtggggtcgg	ISR		Aakra <i>et al.</i> (1999a)
9R	cgtattaccgcggctgct	16S rDNA	519–536*	Lane <i>et al.</i> (1985)
3R	cccgtaattcatttgagtt	16S rDNA	907–926*	Reverse of 16F; Lane <i>et al.</i> (1985)
4R	gacggcggtgtgtga	16S rDNA	1392–1406*	Lane <i>et al.</i> (1985)
5R	ggttacctgttacgactt	16S rDNA	1492–1510*	Stackebrandt & Charfreitag (1990)
ISR-R	gatcacccctgcttgcg	ISR		Aakra <i>et al.</i> (1999a)
13R	gccaaagcatccaccatg	23S rDNA	18–37†	Aakra <i>et al.</i> (1999a)

* *E. coli* numbering according to Brosius *et al.* (1978).

† *E. coli* numbering according to Brosius *et al.* (1980).

Fig. 1. Neighbour-joining distance trees based on 16S rRNA gene sequences (a) and 16S–23S rDNA ISR sequences (b) from AOB in the β -subclass of the *Proteobacteria*. The relative distances were calculated using the DNAST program in the PHYLIP program package (Felsenstein, 1993). Abbreviations of the organisms are given in Table 1. '*Nitrosococcus mobilis*' Nc2 was defined as an outgroup organism in both trees. The various groups of organisms have been named NmA–NmC for the *Nitrosomonas* group and NspA–NspD for the *Nitrospira* group. Accession numbers for the sequences are given in parentheses.

Fig. 2. Majority rule consensus trees of bootstrapped neighbour-joining analyses of 16S rDNA and ISR sequences from AOB, corresponding to the neighbour-joining distance trees in Fig. 1. '*Nitrosococcus mobilis*' Nc2 was defined as an outgroup organism. The clusters are denoted as in Fig. 1. The consensus tree based on the 16S rDNA sequence data are shown on the left (a); the ISR-based tree is shown on the right (b). For the *Nitrosomonas* isolates, the topologies of and differences between the trees are the same as in the trees shown in Fig. 1. Within the *Nitrospira* clade, however, it is less clear. The ISR-based consensus tree displays the same topology as the neighbour-joining distance tree (Fig. 1b), but the consensus tree based on 16S rDNA sequences (a) differs slightly from the corresponding tree in Fig. 1(a). In the 16S rDNA consensus tree (a) the NspA organisms are separated from each other into three clusters, but only one of the branch points leading to this separation is fully supported by the bootstrap analysis.

The 24 *Nitrospira* isolates included in Fig. 1(a) were divided into four clusters, some of them supported by low bootstrap values (Fig. 2a). The similarity values between the *Nitrospira* 16S rRNA genes are high, from 96.3% (between A4 and III2) to 100% (between Ka3 and Ka4). Teske *et al.* (1994) argued that '*Nitrosolobus*', due to its distinct morphological characteristics, should not be included in the *Nitrospira* genus, but in that report only one '*Nitrosolobus*' 16S rDNA sequence was analysed (NI13). In the present study, one new isolate with '*Nitrosolobus*' morphology (NI20) has been analysed by rDNA sequencing. Both the NI13 and NI20 group within the *Nitrospira* clade, but they appear as marginal groups in the 16S rDNA, as well as in the ISR-based tree (discussed below). In the neighbour-joining tree (Fig. 1a) NI20 appears to be most closely related to the organisms of cluster NspA. However, the position of NI20 appears unclear, since it is separated from the other NspA organisms in the majority rule consensus tree (Fig. 2a). In the latter tree, cluster NspA is split into three groups, although two of the branch points

are not statistically significant (bootstrap values 38 and 34%). The NI13 isolate appears most closely related to the NspC organisms (Nsp2 and Nsp17). In the ISR-based tree, the peculiarity of NI13 is less obvious than in the 16S rDNA-based tree, since B6 groups together with NI13 here. However, we do not believe this reflects a true phylogenetic relationship, since the phylogenetic position of B6 probably is within the NspA cluster, as seen both in the 16S rDNA-based tree and in trees based on partial sequences of the ammonia monooxygenase gene (A. Aakra, unpublished results). It is tempting to speculate that the lobate morphology is a trait that, in terms of evolutionary rates, has evolved recently and we recommend that '*Nitrosolobus*' should be kept as a genus alongside *Nitrospira* until more AOB with lobate cell morphology have been examined.

Species affiliations of *Nitrospira* spp. based on DNA-DNA homology values. Previously, DNA-DNA homology values have been obtained for nine of the *Nitrospira* isolates included in this study: NI20 (H.-P. Koops,

unpublished results), N113, Nsp1, Nsp2, Nsp10, Nsp12, Nsp17, Nv1 and Nv6 (Koops & Harms, 1985). According to these data, they all belong to different species, despite the high 16S rDNA similarity values that vary between 97.6 and 99.7%. This demonstrates again that 16S rDNA similarity cannot be used to infer species identity. In this work DNA–DNA hybridization experiments have been performed with AF, L115, A4, A16, 24C, 40KI, III2, 39–19, F3, B6 and III7. According to the results of these experiments, 24C, AF, L115, A16 and A4, i.e. the organisms constituting the NspD cluster, belong to the same species (DNA homology values 52–81%), a species that has not been described before. According to the definition of AOB clusters by Stephen *et al.* (1996), the NspD organisms belong to ‘*Nitrosospira* cluster 3’. Likewise, B6 and III7 (DNA homology value 74%) represent a hitherto undescribed *Nitrosospira* species within ‘*Nitrosospira* cluster 2’ (cluster definitions by Stephen *et al.*, 1996). 40KI, III2 and Nsp12 constitute a subgroup within NspA and they belong to the same species (homology values between 68 and 73%). Within the NspB cluster, F3 and Nsp1 appear to belong to one species (homology value 69%) and 39–19 belongs to the same species as Nsp10 (homology value 55%). DNA–DNA hybridization experiments have not been performed with the other *Nitrosospira* isolates, since we have not been able to obtain sufficient cell material from these organisms.

Phylogeny of AOB based on ISR sequences. Based on 12 sequences of the 16S–23S rDNA ISR it has been suggested that phylogenetic analysis of the ISR could be used as a supplementary tool to 16S rDNA sequencing when studying the diversity of AOB (Aakra *et al.*, 1999a). In the present work, the ISRs from 26 more AOB isolates have been amplified, sequenced, aligned and analysed phylogenetically, resulting in the tree shown in Fig. 1(b). The size of the ISR varied from 394 bp in Nm57 and F6, to 708 bp in 24C. Generally, the ISRs from the *Nitrosomonas* and *Nitrosococcus* isolates are shorter (394–618 bp) than the ISRs from the *Nitrosospira* isolates (633–708 bp) (Table 3), but the differences are not sufficient enough to be used for genus recognition, as discussed previously (Aakra *et al.*, 1999a).

Compared to the 16S rRNA gene sequences, the similarity values of the ISR sequences are low. Within the *Nitrosomonas* and *Nitrosococcus* genera, the similarity values vary from 43.0 to 97.0%. For the *Nitrosospira* isolates, the similarity values vary from 65.4% to almost identity (99.4%). This is not merely due to ISR size differences, because those organisms, having equal sized ISRs, have ISR similarity values of 97.0 (F6 and Nm57), 77.6 (Nm33 and Nm41), 97.8 (40KI and Nsp12) and 86.2% (O4 and Ka3). To a certain extent, the similarity of the ISRs can be ascribed to conserved regions of the ISR, because all the AOB isolates examined contain two tRNA genes (tRNA-Ile and tRNA-Ala) near the 5' end of the ISR. The conserved regions of the ISR are nearly identical

Table 3. Length of the new 16S–23S rDNA ISRs (bp)

ISRs of remaining isolates have been presented elsewhere (Aakra *et al.*, 1999a). The clusters are denoted as in Figs 1 and 2.

Isolate	ISR (bp)	Cluster
<i>Nitrosomonas</i> sp. F6	394	NmA
<i>Nitrosomonas eutropha</i> Nm57	394	NmA
‘ <i>Nitrosococcus mobilis</i> ’ Nc2	423	Outgroup organism
<i>Nitrosomonas oligotropha</i> Nm45	497	NmB
<i>Nitrosomonas ureae</i> Nm10	518	NmB
<i>Nitrosomonas communis</i> Nm2	531	NmB
<i>Nitrosomonas</i> sp. Nm33	548	NmB
<i>Nitrosomonas</i> sp. Nm41	548	NmB
<i>Nitrosomonas marina</i> Nm22	566	NmB
<i>Nitrosomonas aestuarii</i> Nm36	596	NmB
<i>Nitrosomonas cryotolerans</i> Nm55	612	NmC
<i>Nitrosomonas</i> sp. Nm51	618	NmB
<i>Nitrosospira tenuis</i> Nv1 ^T	633	NspB
<i>Nitrosospira</i> sp. Nv6	637	NspB
<i>Nitrosospira</i> sp. 39–19	643	NspB
<i>Nitrosospira</i> sp. Nsp2	649	NspC
<i>Nitrosospira</i> sp. Nsp17	654	NspC
<i>Nitrosospira briensis</i> Nsp10	661	NspB
<i>Nitrosospira</i> sp. N120	664	NspA
<i>Nitrosospira</i> sp. Nsp12	674	NspA
<i>Nitrosospira</i> sp. Nsp1	675	NspB
<i>Nitrosospira</i> sp. O13	676	NspA
<i>Nitrosospira</i> sp. Ka3	677	NspA
<i>Nitrosospira</i> sp. O4	677	NspA
<i>Nitrosospira</i> sp. Ka4	678	NspA
<i>Nitrosospira</i> sp. 24C	708	NspD

in all the AOB isolates tested and constitute approximately 200 bp. The heterogeneity of the ISRs of the AOB is largely due to insertions and deletions outside the conserved regions. These differences often include nucleotide blocks, rather than single bp substitutions, which might be useful for possible future construction of specific oligonucleotides to be used for studies of AOB communities.

The phylogeny of AOB based on ISR sequences complements the 16S rDNA-based phylogeny

When comparing phylogenetic trees constructed on the basis of different genes, small topology differences must be expected, since it is not likely that functionally independent markers have preserved information from the same periods of evolution. When comparing the two neighbour-joining trees in Fig. 1, the phylogeny of the 16S rRNA gene sequences and the 16S–23S rDNA ISR, appear consistent. The main difference between the two trees is that the ISR-based tree has a much higher resolution, indicated by the bars reflecting the relative distances (Fig. 1). After the submission of this paper, a comparison of the phylogeny of various AOB,

based on 16S rRNA gene sequences and *amoA* sequences was published (Purkhold *et al.*, 2000). This study, which focuses on *Nitrosomonas* spp. concluded that the evolution of the ammonia monooxygenase gene is similar, though not identical, to that of the 16S rRNA gene. Although the organisms constituting the NmB group in the present paper have been divided into three groups by Purkhold *et al.* (2000), their results are in agreement with the results presented in this paper.

With a few exceptions, the clustering of the *Nitrosomonas* isolates in the ISR tree (Fig. 1a) is the same as in the 16S rDNA tree (Fig. 1b). The *Nitrosomonas* isolates have been divided into three subclusters denoted NmA–C (Figs 1 and 2), and within NmA and NmB some differences can be seen between the 16S rDNA- and the ISR-based trees. Possibly, the differences can be explained by the fast evolution of the ISR compared to the 16S rRNA gene. In the *Nitrosomonas* cluster, differences between the two trees regarding branching patterns are found in NmB, where Nm10 and Nm45 cluster together in the 16S rDNA-based tree (Fig. 1a), whereas they are separated, but still within NmB, in the ISR-based tree (Fig. 1b). In the 16S rDNA-based tree, Nm2 forms its own clade, whereas in the ISR-based tree it clusters with Nm33. Conversely, Nm41 is alone in the ISR tree, but clusters with Nm33 in the 16S tree. The nodes dividing these organisms are all supported by relatively high bootstrap values in the consensus trees (Fig. 2) (59–100%). According to the ISR tree, the separation of the NmB organisms from Nm55 (in NmC) appears to have occurred in two steps, while the 16S rDNA tree suggests one step. In the ISR tree Nm41, Nm2 and Nm33 have, apparently, branched off from the other NmB organisms at an earlier stage in the evolution than is revealed by the 16S rDNA tree (Fig. 1a). This phenomenon may reflect the fact that the ISR reveal more details of the evolution than the functional ribosomal genes do.

The *Nitrosospira* isolates have been divided into four subclusters, denoted NspA–D (Fig. 1b). Among these, NspB, NspC and NspD are almost completely consistent between the 16S rDNA and ISR trees. The clustering of the organisms in subcluster NspA in the 16S rDNA tree is also largely confirmed by the ISR-based phylogeny, although the position of *Nitrosospira* sp. B6 is uncertain. This isolate belongs to NspA in the 16S rDNA-based tree (Fig. 1a), while being more closely related to the NspC organisms in the ISR tree (Fig. 1b).

The statistical significance of the phylogenetic trees in Fig. 1 was tested by bootstrap analyses with 100 replicates (Fig. 2). The majority rule consensus trees (corresponding to the neighbour-joining trees in Fig. 1), with bootstrap values, are shown in Fig. 2. The bootstrap analysis of the 16S rDNA sequence data reveals some uncertainties, particularly concerning the NspA and NspC clusters (see legend to Fig. 2 for

details). Still, all the nodes leading to discrepancies between the neighbour-joining tree in Fig. 1(a) and the majority rule consensus tree in Fig. 2(a) are supported by low bootstrap values and should not be considered significant. The discrepancies between the trees probably reflect the difficulties in performing phylogenetic analyses of highly similar DNA sequences, such as the AOB 16S rDNA sequences, and also the problems of comparing sequences with different evolutionary rates. Possibly, some features of the ISR tree reflect recent evolutionary events that are not yet reflected in the 16S rDNA tree. The main topologies of the two trees based on the ISR sequence data (Figs 1b and 2b) are the same, although some of the nodes separating the *Nitrosospira* clusters appear with low frequencies. Nodes appearing with low frequencies in the 16S rDNA-based consensus tree (Fig. 2a) correspond to nodes with low bootstrap values in the ISR-based consensus tree (Fig. 2b).

Comparison of the new 16S rDNA sequences with oligonucleotides assumed to be specific for the 16S rRNA gene of β -proteobacterial AOB

The match between previously published 16S rDNA sequences and oligonucleotides assumed to be specific for the AOB has been reviewed by Utåker & Nes (1998). Since this review was published, some new AOB-specific oligonucleotides have been reported (Stephen *et al.*, 1998). An alignment between the new 16S rDNA sequences and the published AOB-specific oligonucleotides was performed to check the match between the new 16S rDNA sequences and these oligonucleotides.

In general, the new sequences presented here do not alter the claimed specificity of the 31 AOB-specific oligonucleotides evaluated by Utåker & Nes (1998). Comparison between the 16S rDNA sequences presented in this study and the 16S rDNA-specific oligonucleotides constructed by Stephen *et al.* (1998) show some discrepancies, however. The oligonucleotide named NspCL1_249, is not 100% complementary to any of the 38 16S rDNA sequences. The NspCL1_249 sequence is assumed to be specific for organisms in the '*Nitrosospira* cluster 1' (cluster definitions by Stephen *et al.*, 1996), from which no pure cultures of AOB are available. Consequently, the quality and biological significance of this oligonucleotide is more difficult to assess. The best match between the NspCL1_249 oligonucleotide and a 16S rDNA sequence from a pure culture is obtained with *Nitrosomonas* sp. Nm33 (two mismatches), while there are three or four mismatches with the other 37 sequences included in the present study. The specificity of the *Nitrosomonas*-specific oligonucleotides Nmo254 and Nmo254a is not convincing in distinguishing well between the *Nitrosospira*- and *Nitrosomonas*-like sequences. Unless very stringent hybridization conditions are used, it is likely that Nmo254 and Nmo254a will detect most β -subclass *Proteobacteria*. The same

may be true for NmoCL6a_205 which has three mismatches with several *Nitrosomonas* sequences and with all *Nitrospira* sequences. Most of the mismatches between these oligonucleotides and the 16S rDNA sequences are found in or near the middle of the sequences, which might be problematic, especially if the oligonucleotides should be used for specific detection of certain groups of AOB.

Conclusion

In the present work information on the phylogeny of AOB based on the 16S rRNA genes and the 16S–23S rDNA ISRs has been obtained. Phylogenetic trees based on these two regions display high consistency regarding the relationships between the organisms included. Although the DNA–DNA homology values have not been determined for all the organisms studied here, the patterns shown are good indications that species affiliations of AOB based on DNA–DNA homologies are in accordance with the phylogenetic relations based on rDNA sequences. Species determination based on DNA–DNA hybridization is a technique that is the ‘standard arbiter’ for species designation (Murray *et al.*, 1990; Stackebrandt & Goebel, 1994), but the technique is laborious and demands relatively large amounts of cell material which are difficult (and sometimes impossible) to obtain with AOB. Although the species determination by DNA homology values is important, particularly for organisms that are closely related at the 16S rRNA gene level, the term ‘rRNA species complex’ (Stackebrandt & Goebel, 1994) could be an applicable alternative. In our opinion, the data obtained here are compelling evidence that the 16S rDNA, the 16S–23S rDNA ISR sequences and species affiliations reflect the same relationships between AOB, which is important information for future analyses of natural diversity of AOB.

Since the ISR and 16S rDNA sequences of AOB display consistent phylogenies, and since the ISR sequences show considerably higher variability than the 16S rDNA sequences from the same organisms, the importance of the ISR for molecular ecology studies is clear. Alignment of the ISR sequences from the AOB revealed several regions that probably can be used as oligonucleotides specific for all or for certain subgroups of the AOB in the β -subclass of the *Proteobacteria*. In fact, the variability of ISR sequences from AOB might be so high that this region could be used in ecological applications. Thus, for assessment of natural populations of AOB, possible specific oligonucleotides based on ISR sequences might be used in combination with specific oligonucleotides based on 16S rDNA sequences.

ACKNOWLEDGEMENTS

The authors thank Dr Jenny Norton, Utah State University, for kindly providing the *Nitrospira* isolates 24C and 39–19. This work was supported by grants from the Norwegian Research Council to J.B.U. and to A.A.

REFERENCES

- Aakra, Å., Utåker, J. B. & Nes, I. F. (1999a). RFLP of rRNA genes and sequencing of the 16S–23S rDNA intergenic spacer region of ammonia oxidizing bacteria: a phylogenetic approach. *Int J Syst Bacteriol* **49**, 123–130.
- Aakra, Å., Utåker, J. B., Nes, I. F. & Bakken, L. (1999b). An evaluated improvement of the extinction dilution method for isolation of ammonia-oxidizing bacteria. *J Microbiol Methods* **39**, 23–31.
- Aakra, Å., Hesselsoe, M. & Bakken, L. R. (2000). Surface attachment of ammonia-oxidizing bacteria in soil. *Microb Ecol* **39**, 222–235.
- Acinas, S. G., Anton, J. & Rodriguez-Valera, F. (1999). Diversity of free-living and attached bacteria in offshore Western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA. *Appl Environ Microbiol* **65**, 514–522.
- Barry, T., Collieran, G., Glennon, M., Dunican, L. K. & Gannon, F. (1991). The 16S/23S ribosomal spacer region as a target for DNA probes to identify eubacteria. *PCR Methods Appl* **1**, 51–56.
- Borneman, J. & Triplett, E. W. (1997). Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Appl Environ Microbiol* **63**, 2647–2653.
- Brosius, J., Palmer, M. L., Kennedy, P. J. & Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci USA* **75**, 4801–4805.
- Brosius, J., Dull, T. J. & Noller, H. F. (1980). Complete nucleotide sequence of a 23S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci USA* **77**, 201–204.
- Edwards, U., Rogall, T., Blocker, H., Emde, M. & Bottger, E. C. (1989). Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* **17**, 7843–7853.
- Felsenstein, J. (1993). PHYLIP (Phylogeny Inference Package), 3.572c. Seattle: Department of Genetics, University of Washington.
- Fox, G. E., Wisotzkey, J. D. & Jurtshuk, P., Jr (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* **42**, 166–170.
- Garcia-Martinez, J., Acinas, S. G., Anton, A. I. & Rodriguez-Valera, F. (1999). Use of the 16S–23S ribosomal genes spacer region in studies of prokaryotic diversity. *J Microbiol Methods* **36**, 55–64.
- Gürtler, V. & Stanisich, V. A. (1996). New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. *Microbiology* **142**, 3–16.
- Head, I. M., Hiorns, W. D., Embley, T. M., McCarthy, A. J. & Saunders, J. R. (1993). The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. *J Gen Microbiol* **139**, 1147–1153.
- Head, I. M., Hiorns, W. D., Embley, T. M., McCarthy, A. J. & Saunders, J. R. (1995). *Nitrospira multiformis* comb. nov. and *Nitrospira tenuis* comb. nov. In *Validation of the Publication of New Names and New Combinations Previously Effectively Published Outside the IJSB*, List no. 54. *Int J Syst Bacteriol* **45**, 619–620.
- Jensen, M. A., Webster, J. A. & Straus, N. (1993). Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl Environ Microbiol* **59**, 945–952.

- Jiang, Q. Q. & Bakken, L. R. (1999). Comparison of *Nitrospira* strains isolated from terrestrial environments. *FEMS Microbiol Ecol* **30**, 171–186.
- Jones, R. D., Morita, R. Y., Koops, H.-P. & Watson, S. W. (1988). A new marine ammonium-oxidizing bacterium, *Nitrosomonas cryotolerans* sp. nov. *Can J Microbiol* **34**, 1122–1128.
- Koops, H.-P. & Harms, H. (1985). Deoxyribonucleic acid homologies among 96 strains of ammonia-oxidizing bacteria. *Arch Microbiol* **141**, 214–218.
- Koops, H.-P., Böttcher, B., Möller, U. C., Pommerening-Röser, A. & Stehr, G. (1991). Classification of eight new species of ammonia-oxidizing bacteria: *Nitrosomonas communis* sp. nov., *Nitrosomonas ureae* sp. nov., *Nitrosomonas aestuarii* sp. nov., *Nitrosomonas marina* sp. nov., *Nitrosomonas nitrosa* sp. nov., *Nitrosomonas eutropha* sp. nov., *Nitrosomonas oligotropha* sp. nov. and *Nitrosomonas halophila* sp. nov. *J Gen Microbiol* **137**, 1689–1699.
- Kowalchuk, G. A., Stephen, J. R., de Boer, W., Prosser, J. I., Embley, T. M. & Woldendorp, J. W. (1997). Analysis of ammonia-oxidizing bacteria of the β subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. *Appl Environ Microbiol* **63**, 1489–1497.
- Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. & Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci USA* **82**, 6955–6959.
- McCaig, A. E., Embley, T. M. & Prosser, J. I. (1994). Molecular analysis of enrichment cultures of marine ammonia oxidisers. *FEMS Microbiol Lett* **120**, 363–368.
- Murray, R. G. E., Brenner, D. J., Colwell, R. R., deVos, P., Goodfellow, M., Grimont, P. A. D., Pfennig, N., Stackebrandt, E. & Zavarzin, G. A. (1990). Report of the ad hoc committee on approaches to taxonomy within the *Proteobacteria*. *Int J Syst Bacteriol* **40**, 213–215.
- Nagpal, M. L., Fox, K. F. & Fox, A. (1998). Utility of 16S–23S rRNA spacer region methodology: how similar are interspace regions within a genome and between strains for closely related organisms? *J Microbiol Methods* **33**, 211–219.
- Page, R. D. M. (1996). TreeView: An application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.
- Pommerening-Röser, A., Rath, G. & Koops, H.-P. (1996). Phylogenetic diversity within the genus *Nitrosomonas*. *Syst Appl Microbiol* **19**, 344–351.
- Purkhold, U., Pommerening-Röser, A., Juretschko, S., Schmid, M. C., Koops, H. P. & Wagner, M. (2000). Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys. *Appl Environ Microbiol* **66**, 5368–5382.
- Robledo, E. A., Borneman, J. & Triplett, E. W. (1998). Effects of bacterial antibiotic production on rhizosphere microbial communities from a culture-independent perspective. *Appl Environ Microbiol* **64**, 5020–5022.
- Stackebrandt, E. & Charfreitag, O. (1990). Partial 16S rRNA primary structure of five *Actinomyces* species: phylogenetic implications and development of an *Actinomyces israelii* specific oligonucleotide probe. *J Gen Microbiol* **136**, 37–43.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Stephen, J. R., McCaig, A. E., Smith, Z., Prosser, J. I. & Embley, T. M. (1996). Molecular diversity of soil and marine 16S rRNA gene sequences related to β -subgroup ammonia-oxidizing bacteria. *Appl Environ Microbiol* **62**, 4147–4154.
- Stephen, J. R., Kowalchuk, G. A., Bruns, M. A. V., McCaig, A. E., Phillips, C. J., Embley, T. M. & Prosser, J. I. (1998). Analysis of beta-subgroup proteobacterial ammonia oxidizer populations in soil by denaturing gradient gel electrophoresis analysis and hierarchical phylogenetic probing. *Appl Environ Microbiol* **64**, 2958–2965.
- Teske, A., Alm, E., Regan, J. M., Toze, S., Rittmann, B. E. & Stahl, D. A. (1994). Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. *J Bacteriol* **176**, 6623–6630.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Utåker, J. B. & Nes, I. F. (1998). A qualitative evaluation of the published oligonucleotides specific for the 16S rDNA gene sequences of the ammonia-oxidizing bacteria. *Syst Appl Microbiol* **21**, 72–88.
- Utåker, J. B., Bakken, L., Jiang, Q. Q. & Nes, I. F. (1995). Phylogenetic analysis of seven new isolates of ammonia-oxidizing bacteria based on 16S rRNA gene sequences. *Syst Appl Microbiol* **18**, 549–559.
- Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.