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A Qualitative Evaluation of the Published Oligonucleotides Specific for the 16S rRNA Gene Sequences of the Ammonia-oxidizing Bacteria

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Summary

Over the past few years, there has been an increasing interest in making oligonucleotides specific for ammonia-oxidizing bacteria (AOB), in order to detect and monitor these slow growing bacteria in environmental samples, in enrichment cultures and in wastewater treatment plants. Based on 16S rDNA sequences, a broad selection of oligonucleotides have been designed, either encompassing all known AOB in the β-subgroup of the *Proteobacteria* (βAOB), or subclasses within βAOB. Thirty different oligonucleotides have so far been published, with varying specificity. The first AOB-specific oligonucleotides published were obtained as a result of an alignment of only eleven 16S rDNA sequences from AOB. Including the present study, there are now forty nearly full length 16S rDNA sequences available from these bacteria, in addition to a number of partial sequences, so that an improved evaluation of the published oligonucleotides can be done. Two new 16S rRNA gene sequences from *Nitrosospira* are presented here, in a phylogenetic analysis containing every 16S rRNA gene sequences (>1 kb) available from AOB. On the basis of an alignment of all these sequences, combined with searches in the nucleotide sequence databases, an evaluation of the thirty published oligonucleotides is presented. The analysis expose the strength and weakness of each oligonucleotide and discuss the use of oligonucleotides specific for 16S rRNA genes in future studies of AOB. The present work also identifies one new, broad range primer, specific for the AOB in the β-subgroup of the *Proteobacteria*.

Key words: ammonia-oxidizing bacteria – nitrifying bacteria – specific oligonucleotides – PCR – 16S rRNA – phylogeny

Introduction

The ammonia-oxidizing bacteria (AOB) are a group of autotrophic, Gram negative bacteria, responsible for the first oxidation step in the nitrification process (WATSON et al., 1989). The AOB have until recently been regarded as strict aerobes, but anaerobic growth on ammonia has now been proved for one strain of AOB (SCHMIDT and BOCK, 1997). AOB consist of three different genera; *Nitrosomonas, Nitrosococcus* and *Nitrosospira* (HEAD et al., 1993; TESKE et al., 1994), which all belong to the β-subgroup of the *Proteobacteria* (βAOB), except for *Nitrosococcus oceani* (nom. corrig.; TRÜPER and DE'CLARI, 1997) which is confined to the γ-subgroup (γAOB) (WOESE et al., 1984; WOESE et al., 1985). The AOB comprise five distinct morphology groups, previously used as the basis for their genus classification. Because of their close phylogenetic relation to the *Nitrosospira* genus, however, the genera *Nitrosolobus* and *Nitrosovibrio* have been reclassified as *Nitrosospira* (HEAD et al., 1993). *Nitrosolobus* and *Nitrosovibrio* are now considered as basonymes for those *Nitrosospira* spp. which have a lobate or vibrio morphology, respectively (Int. J. Syst. Bacteriol., List No. 54). The AOB are extremely slow growing, and are difficult to isolate and grow in pure culture (KOOPS and HARMS, 1985; WATSON et al., 1989). Hence, a relatively small number of AOB species have been isolated and characterized so far.

Non-standard abbreviations: AOB – ammonia-oxidizing bacteria; βAOB – ammonia-oxidizing bacteria belonging to the β-subgroup of the *Proteobacteria*; γAOB – ammonia-oxidizing bacteria belonging to the γ-subgroup of the *Proteobacteria*; AMO – ammonia monooxygenase; HAO – hydroxylamine oxidoreductase; T – type strain

Oligonucleotides specific for the ammonia-oxidizers' 16S rRNA genes have been shown to be a promising tool for the recognition and detection of unknown, and perhaps unculturable species of AOB (McCaig et al., 1994; Voytek and Ward, 1995a and b; Hiorns et al., 1995; Wagner et al., 1995; Mobarry et al., 1996; Stephen et al., 1996; Hovanec and DeLong, 1996; Pommerening-Röser et al., 1996; Chandler et al., 1997; Hastings et al., 1997; Kowalchuk et al., 1997; Ward et al., 1997). The AOB-specific oligonucleotides have been used in different ways: in PCR on extracted total DNA, in hybridization to extracted total RNA, and in whole cell hybridization or *in situ* hybridization in both mixed cultures and environmental samples. In activated sludge, the use of oligonucleotides is important for a fast and specific identification of nitrifiers, whose activity is the rate limiting step in the removal of nitrogen from sewage (Wagner, 1995).

The application of specific oligonucleotides for the detection of bacterial strains in the environment has some limitations which must be considered (Stahl and Amann, 1991; Amann et al., 1995; Amann et al., 1996). Most importantly, the majority of oligonucleotides are made on the basis of a relatively small number of DNA sequences, usually originating from pure cultures, which in most cases are not representative of the true diversity of bacteria in the environment (Wagner et al., 1993). This is particularly evident for the AOB, because of the low number of pure cultures available from these bacteria. Consequently, there is always a risk that the oligonucleotides fail to discriminate between specific and unspecific targets (Amann et al., 1995 and 1996). Aside from this, the use of specific oligonucleotides is a powerful tool for the qualitative detection and identification of specific bacteria in environmental samples. Oligonucleotide quality is important, but the *techniques* in which the oligonucleotides are used, can also be limiting: both PCR analysis and hybridization analysis of total RNA are dependent on the cell lysis efficiency and may in addition be biased by the presence of extracellular DNA or RNA. A PCR analysis is also dependent on the fidelity of the *Taq*-polymerase, and the quality of the template molecule (Amann et al., 1995; Kopczynski et al., 1994). The least biased way of investigating bacteria in environmental samples is therefore probably *in situ* hybridization (Wagner et al., 1993; Amann et al., 1995 and 1996). This technique is brilliant for studies of bacteria in their normal habitat, and allows the enumeration of a specific type of bacteria in environmental samples. However, to detect unknown sequences of e. g. 16S rDNA, it will probably be more appropriate to use PCR, which is less specific.

Over the past four years, thirty oligonucleotides specific for the 16S rRNA genes of the AOB have been published. At the same time, the number of available 16S rRNA gene sequences (partly and full length) has increased, often with a concomitant change in the quality of the published oligonucleotides. It is therefore necessary to perform comprehensive database searches and sequence alignments to select which oligonucleotides to use for the detection of AOB. The present study undertakes a critical evaluation of the available oligonucleotides, which hopefully will make it easier to perform this selection.

All the reported oligonucleotides recognizing AOB-16S rDNA have so far been designed on the basis of 16S rDNA sequences from pure cultures of AOB. As already mentioned, these sequences most probably do not reflect the actual abundance of AOB in the environment (Mobarry et al., 1996; Pommerening-Röser et al., 1996). Recently, however, a number of new 16S rDNA sequences have been reported, obtained by direct amplification from environmental samplex, and shown to have high sequence similarities with the 16S rDNA from βAOB (Stephen et al., 1996; Kowalchuk et al., 1997). Although comparative 16S rDNA analysis does not allow an unambigous assignment of hitherto uncultured microorganisms to a distinct physiological group, it is probable that these sequences originate from a group of AOB not biased by the selection that occurs in a normal enrichment procedure. As the number of such 16S rDNA sequences increases, the design of specific oligonucleotides and the interpretation of results from their use will be more adequate.

The present study report two new 16S rRNA gene sequences from pure cultures of *Nitrosospira*, and present one new, βAOB-specific oligonucleotide. An alignment of the forty available 16S rRNA genes from AOB is used as a basis for an evaluation of the previously published oligonucleotides, reported to be specific for the βAOB.

Materials and Methods

Pure cultures of AOB: Enrichment cultures were set up as described previously (Utåker et al., 1995; Jiang et al., 1996), and the isolated, pure cultures were grown at room temperature in a dilute ammonium medium supplemented with trace elements (Donaldson and Henderson, 1989; Schmidt and Belser, 1994).

DNA extraction: Cells were lysed by the method described by Head et al. (1993), except for an increased number of freeze thaw cycles and the use of liquid N_2 instead of dry ice. In short, 50 ml of a pure culture with a cell density of between $1 \cdot 10^6$ and $1 \cdot 10^7$ cells/ml was pelleted, and washed twice in 2 ml 1×TE-buffer (pH 8.0), followed by resuspension in 50 µl lysis-buffer [1×TE (pH 8.0)], supplemented with 1% Tween 80 (Sigma Chemical Co., St. Louis, USA). Samples were snap frozen in liquid N_2, followed by thawing on a boiling water bath. This freeze and thaw cycling was repeated four times. Cell debris was pelleted and the supernatant was subjected to PCR-amplification directly.

Amplification and sequencing of 16S rDNA: Nearly full length 16S rDNA was amplified by PCR, using conditions and bacterial primers as described previously (Utåker et al., 1995). Amplified DNA was sequenced in both directions, using the same primers and procedure as described previously (Utåker et al., 1995).

The βAOB specific primers AMβ (this study) and NitB (Voytek and Ward, 1995a) were tested on pure cultures of AOB. 1 µl cell lysate (prepared as described above) was used as a DNA template under the following PCR conditions: 200 µM of each nucleotide, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 1% formamide, 0.025 U/µl of *Taq* polymerase (Advanced Biotechnologies Ltd.,

London, UK). Amplifications were performed in a Perkin-Elmer/Cetus DNA thermal cycler (Norwalk, Connecticut, USA) with cycling conditions as follows: hot start for 3 min at 97 °C, followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 69 °C and extension for 1 min at 72 °C.

Data analysis: Nucleotide sequences were analyzed in the GCG program package, ver. 8.1 [Program Manual for the Wisconsin Package, Version 8, August 1994; Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711], and the Extended GCG program package, ver. 8.1 [Program manual for the EGCG Package; Peter Rice, The Sanger Centre, Hinxton Hall, Cambridge, CB 10 1RQ, England]. Sequence alignment was done using the ClustalW multiple alignment program (THOMPSON et al., 1994) in GCG. This alignment was the basis for a phylogenetic tree construction, using programs in the Phylip program package, ver. 3.5c (FELSENSTEIN, 1993): DNADIST (KIMURA, 1980), NEIGHBOR (SAITOU and NEI, 1987), DNAPARS (FELSENSTEIN, 1993), SEQBOOT (FELSENSTEIN, 1985) and CONSENSE (FELSENSTEIN, 1993). Sequences used for alignment and phylogenetic analysis were all adjusted to the length of the shortest 16S rDNA sequence in the analysis – which was 1045 nt. Phylogenetic trees were visualized using the Tree View© software (PAGE, 1996).

By searching the nucleotide sequence databases using the two programs FASTA (SMITH and WATERMAN, 1981) and MPSearch (PEARSON and LIPMAN, 1988), the number of potential, non-specific target sequences for each of the specific oligonucleotides was registered.

The program packages GCG, EGCG, ClustalW and Phylip were all run on a UNIX mainframe computer.

Nucleotide sequence accession numbers: The 16S rDNA sequences used for alignment and phylogenetic tree construction were fetched from the EMBL database (per 010497), under the following accession numbers (organisms in alphabetical order): *Escherichia coli*, acc. no. J01859; *Nitrosococcus mobilis* Nc2^T, acc. no. M96403; *Nitrosococcus oceani* Nc9, acc. no. M96398; *Nitrosococcus oceani* Nc10^T, acc. no. M96395; *Nitrosomonas* sp. Nm63, acc. no. M96400; *Nitrosomonas communis* Nm2^T, acc. no. Z46981; *Nitrosomonas cryotolerans* Nm55^T, acc. no. Z46984; *Nitrosomonas europaea* Nm50^T, acc. no. M96399; *Nitrosomonas eutropha* Nm57^T, acc. no. M96402; *Nitrosomonas halophila* Nm1^T, acc. no. Z46987; *Nitrosomonas marina* Nm22^T, acc. no. Z46990; *Nitrosomonas ureae* Nm10^T, acc. no. Z46993; *Nitrosospira* sp. 40KI, acc. no. X84656; *Nitrosospira* sp. AF, acc. no. X84658; *Nitrosospira* sp. AHB1, acc. no. X90820; *Nitrosospira* sp. B6, acc. no. X84657; *Nitrosospira* sp. C-141, acc. no. M96397; *Nitrosospira* sp. D11, acc. no. X84660; *Nitrosospira* sp. GM4, acc. no. X84659; *Nitrosospira* sp. L115, acc. no. X84662; *Nitrosospira* sp. NpAV, acc. no. Y10127; *Nitrosospira* sp. Np22–21, acc. no. Y10128; *Nitrosospira* sp. Nv12, acc. no. M96405; *Nitrosospira* sp. C-128, acc. no. L35505; *Nitrosospira multiformis* N113^T, acc. no. L35509; *Nitrosospira tenuis* Nv1, acc. no. M96404; β Proteobacterium EnrichZD5, acc. no. Z69146; β Proteobacterium EnvA2–4, acc. no. Z69094; β Proteobacterium EnvA1–21, acc. no. Z69091; β Proteobacterium EnvA2–13, acc. no. Z69097; β Proteobacterium EnvB1–17, acc. no. Z69104; β Proteobacterium EnvC2–23, acc. no. Z69125; β Proteobacterium pH4.2A/23, acc. no. Z69151; β Proteobacterium pH4.2A/3E, acc. no. Z69155; β Proteobacterium pH4.2A/3F, acc. no. Z69156; β Proteobacterium pH4.2A/H2, acc. no. Z69165; β Proteobacterium pH4.2A/G2, acc. no. Z69164; β Proteobacterium pH7B/D3, acc. no. Z69177; β Proteobacterium pH7C/56, acc. no. Z69197.

Accession numbers for the two new 16S rDNA sequences are:
Nitrosospira sp. isolate III2, EMBL acc. no. AJ000344
Nitrosospira sp. isolate III7, EMBL acc. no. AJ000345

Results and Discussion

Phylogeny of the AOB, based on 16S rDNA sequence comparison

The 16S rDNA sequences from two new *Nitrosospira* spp., isolates III2 and III7, are presented in this study. Both isolates originate from organic forest litter, and have a typical spiral morphology (JIANG, 1996). The 16S rRNA genes were sequenced in both directions, giving a nearly full length sequence (approximately 1400 nt.). These sequences were aligned with the available full length 16S rDNA sequences (>1 kb) from AOB, and the alignment was used to construct a phylogenetic distance tree (Figure 1). A number of shorter 16S rDNA sequences from AOB are also available in the databases, but these sequences are too short to give a good phylogenetic comparison, a fact which is particularly evident for the closely related AOB. The phylogenetic tree in Figure 1 emphasized the conclusions in earlier studies: the βAOB are very closely related, and the 16S rDNA based phylogeny has too low resolution to give a good description of *how* these strains and isolates are related (HEAD et al., 1993; TESKE et al., 1994; UTÅKER et al., 1995; POMMERENING-RÖSER et al., 1996). A bootstrapped parsimony tree (not shown), based on the same alignment, confirmed the main branches in the distance tree, but the branching among the closest related taxons is not convincing, as previously reported (UTÅKER et al., 1995).

Design of a new broad range PCR primer, specific for the majority of 16S rDNA molecules from βAOB

Although too conserved to fully describe the phylogeny of the closely related βAOB, the 16S rDNA sequences have been used to construct specific oligonucleotides for the detection of these bacteria in environmental samples, enrichment cultures or wastewater treatment plants. It is possible to make oligonucleotides specific for the whole group of βAOB and for its main subclasses, but the high smiliarity between the 16S rRNA gene sequences makes it difficult to design more specific oligonucleotides, e. g. for the detection of individual morphology groups within the AOB.

The alignment of the forty 16S rDNA-sequences (>1 kb) from different AOB was the basis for the design of a new forward primer, which was designated Amβ [pos. 738–758 in 16S rDNA; *E. coli*-numbering (BROSIUS et al., 1978)]. Compared to other AOB specific primers that have been reported, the specificity of the Amβ primer is reasonably good. A comparison between the available 16S rDNA sequences (>1 kb) from βAOB and the AMβ sequence is shown in Figure 2d. Except for one environmental 16S rDNA sequence (pH4.2A/H2) (STEPHEN et al., 1996), it is likely that the AMβ primer will amplify all these sequences. Because of the mismatches with the environmental sequence, however, it is possible that the Amβ primer may fail to amplify some unknown 16S rDNA sequences from βAOB. Notably, there is a relatively deep branching between the pH4.2/H2-sequence

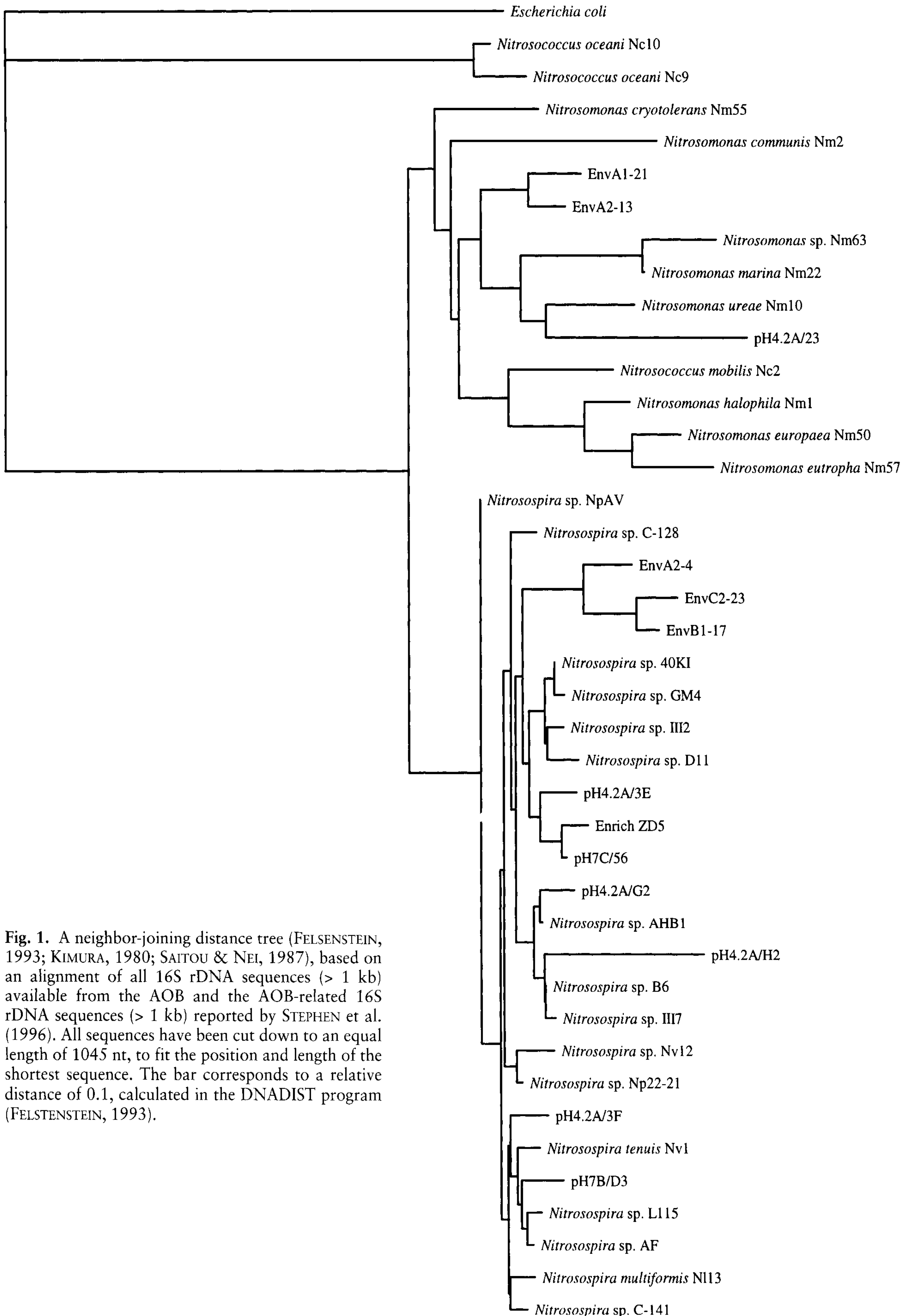

Fig. 1. A neighbor-joining distance tree (FELSENSTEIN, 1993; KIMURA, 1980; SAITOU & NEI, 1987), based on an alignment of all 16S rDNA sequences (> 1 kb) available from the AOB and the AOB-related 16S rDNA sequences (> 1 kb) reported by STEPHEN et al. (1996). All sequences have been cut down to an equal length of 1045 nt, to fit the position and length of the shortest sequence. The bar corresponds to a relative distance of 0.1, calculated in the DNADIST program (FELSTENSTEIN, 1993).

and the other *Nitrosospira*-sequences (Figure 1), and four of the oligonucleotides published by other groups (described later) also fails to recognize this particular 16S rDNA sequence.

A database search with the Amβ sequence shows that it is identical to 16S rDNA from βAOB only, and that the γAOB will not be amplified (six mismatches). However, the Amβ primer has only one mismatch with the 16S rDNA from a number of other bacteria, e. g. the marine genus *Alteromonas* (γ-subgroup of the *Proteobacteria*) and the closely related fresh water bacterium *Gallionella ferruginea* (β-subgroup of the *Proteobacteria*). It is therefore advisable not to use the Amβ primer in combination with primers specific for the same non-AOB organisms. The Amβ primer was successfully tested in a PCR on DNA from pure cultures on AOB (*Nitrosospira* spp. and *Nitrosomonas europaea)*, in combination with the reverse primer NitB (VOYTEK and WARD, 1995a) (results not shown). DNA from the closely related soil bacterium *Ralstonia eutropha* (basonym *Alcaligenes eutrophus*) (β-subgroup of the *Proteobacteria*) was used as a negative control (four mismatches with AMβ and one mismatch with NitB).

Because the AMβ primer is positioned in the middle of the 16S rRNA gene, it is perhaps most useful as a forward primer in the second amplification of a nested PCR, or as an internal probe for the control of PCR products amplified by other, βAOB specific primers.

Evaluation of the published βAOB-specific oligonucleotides

In addition to select a new primer, we wanted to evaluate the published oligonucleotides reported to be specific for the 16S rRNA genes of the βAOB. An overview of these oligonucleotides and their reported specificity is presented in Table 1. The evaluation performed in the present study is mainly based on sequence comparisons and nucleotide database searches, which of course is less informative than a true PCR- or hybridization study would be. A comparison of the oligonucleotides to the alignment of the forty 16S rDNA sequences does, howev-

Table 1. An overview of the thirty-one oligonucleotides published up today, specific for the AOB in the β-subgroup of the *Proteobacteria*, and subgroups within these bacteria.
[1] Positions in the 16S rDNA molecule are given in *E. coli* numbering (BROSIUS et al., 1978).
[2] The specificity listed is according to what the authors claimed at the time the oligonucleotides were published.

Oligo-nucleotide	Position in 16S rDNA[1]	Specificity[2]	Figure	Reference
NM-75	67–86	terrestrial *Nitrosomonas* spp./*Nitrosococcus mobilis*	4 a	HIORNS et al. (1995)
NS-85	76–95	*Nitrosospira* spp.	3	HIORNS et al. (1995)
NmII	120–139	*Nitrosomonas communis*-lineage	5 a	POMMERENING-RÖSER et al. (1996)
NitA	136–158	βAOB	2 a	VOYTEK & WARD (1995a)
βAMOf	142–162	βAOB	2 a	MCCAIG et al. (1994)
Nm0	148–165	*Nitrosomonas* spp.	4 a	POMMERENING-RÖSER et al. (1996)
Nsm156	155–173	*Nitrosomonas* spp./*Nitrosococcus mobilis*	4 a	MOBARRY et al. (1996)
NmV	174–191	*Nitrosococcus mobilis*	5 a	POMMERENING-RÖSER et al. (1996)
Nso190	189–207	βAOB	2 a	MOBARRY et al. (1996)
CTO189f	189–207	βAOB	2 a	KOWALCHUK et al. (1997)
NmI	210–225	*Nitrosomonas europaea*-lineage	5 a	POMMERENING-RÖSER et al. (1996)
AAO258	258–277	terrestrial βAOB	2 c	HIORNS et al. (1995)
NitD	439–461	*Nitrosomonas europaea*	5 a	WARD et al. (1997)
Nsv443	443–461	*Nitrosospira* spp.	3	MOBARRY et al. (1996)
Nsp0	452–469	*Nitrosospira* spp.	3	POMMERENING-RÖSER et al. (1996)
Nlm459r	458–477	*Nitrosospira multiformis*/*Nitrosospira* sp. C-141	3	HASTINGS et al. (1997)
NSM1B	478—494	*Nitrosomonas europaea*-lineage/ *Nitrosococcus mobilis*	5 b	HOVANEC & DELONG (1996)
TAO_{rev}	632–649	βAOB	2 c	CHANDLER et al. (1997)
CTO654r	632–653	βAOB	2 c	KOWALCHUK et al. (1997)
NITROSO4E	638–657	βAOB	2 c	HOVANEC & DELONG (1996)
NEU	651–668	most halophilic and halotolerant *Nitrosomonas*	5 b	WAGNER et al. (1995)
Amβ	738–758	βAOB	2 d	this paper
NitF	844–862	βAOB	2 d	WARD et al. (1997)
NitC	846–862	βAOB	2 d	VOYTEK & WARD (1995a and b)
NmIII	998–1018	*Nitrosomonas marina*-lineage	5 b	POMMERENING-RÖSER et al. (1996)
RNM-1007	1005–1028	terrestrial *Nitrosomonas* spp.	4 b	HIORNS et al. (1995)
NS-1009	1007–1026	*Nitrosospira* spp.	3	HIRNS et al. (1995)
NmIV	1004–1022	*Nitrosomonas cryotolerans*-lineage	5 b	POMMERENING-RÖSER et al. (1996)
NitB	1213–1233	βAOB	2 d	VOYTEK & WARD (1995a)
Nso1225	1224–1243	βAOB	2 d	MOBARRY et al. (1996 and 1997)
βAMOr	1295–1314	βAOB	2 b	MCCAIG et al. (1994)

a)

	136–158	142–162	189–207
Nitrosospira sp. 40KI	-----------G-----------	---------------------	-------------------
Nitrosospira sp. III2	-----------G-----------	---------------------	-------------------
Nitrosospira sp. III7	-----------G-----------	---------------------	-------------------
Nitrosospira sp. AF	T-CG-------G--------C-N	---------------------	-------------------
Nitrosospira sp. AHB1	-----------G-----------	---------------------	-------------------
Nitrosospira sp. B6	-----------G-----------	---------------------	-------------------
Nitrosospira sp. C-128	T-C--------G--------C--	---------------------	-------------------
Nitrosospira sp. D11	-----------G-----------	---------------------	-------------------
Nitrosospira sp. GM4	-----------G-----------	---------------------	-------------------
Nitrosospira sp. L115	T-CG-------G--------C--	---------------------	-------------------
Nitrosospira sp. NpAV	T-CG-------G--------C--	---------------------	-------------------
Nitrosospira sp. Np22-21	T-C--------G--------C--	---------------------	-------------------
Nitrosospira multiformis Nl13	-----------G--------C--	---------------------	-------------------
Nitrosospira sp. C-141	T-CG-------G--------C--	---------------------	-------------------
Nitrosospira tenuis Nv1	T-CG-------G--------C--	---------------------	-------------------
Nitrosospira sp. Nv12	T-C--------G--------C--	---------------------	-------------------
Nitrosomonas sp. Nm63	-A-G-------G-----------	---------------------	-------U-----------
Nitrosomonas communis Nm2	T-A--------G-----------	---------------------	----G--------------
Nitrosomonas cryotolerans Nm55	-----------G-----------	---------------------	-------------------
Nitrosomonas europaea Nm50	-----------------------	---------------------	-------------------
Nitrosomonas eutropha Nm57	-----------------------	---------------------	----G--------------
Nitrosomonas halophila Nm1	-----------------------	---------------------	-------------------
Nitrosomonas marina Nm22	-AAG-N-----G-----------	---------------------	-------------------
Nitrosomonas ureae Nm10	-----------G-----------	---------------------	----G----UG--------
Nitrosococcus mobilis Nc2	-----------G------NNNNN	------------NNNNN----	-------------------
	5' **CTTAAGTGGGGAATAACGCATCG**	**TGGGGRATAACGCAYCGAAAG**	**GGAGAAAAGCAGGGGAUCG** 3'
	NitA (fwd)	*βAMOf (fwd)*	|||||||||||||||||||
			CCTCTTTTCGTCCCCTAGC
			***Nso190** (rev)*
			....R..............
			***CTO189fA/B-GC** (fwd)*
			....G....T.........
			***CTO189fC-GC** (fwd)*

b)

	1295–1314
Nitrosospira sp. 40KI	--------------------
Nitrosospira sp. III2	--------------------
Nitrosospira sp. III7	--------------------
Nitrosospira sp. AF	--------------------
Nitrosospira sp. AHB1	--------------------
Nitrosospira sp. B6	--------------------
Nitrosospira sp. C-128	--------------------
Nitrosospira sp. D11	--------------------
Nitrosospira sp. GM4	--------------------
Nitrosospira sp. L115	--------------------
Nitrosospira sp. NpAV	--------------------
Nitrosospira sp. Np22-21	--------------------
Nitrosospira multiformis Nl13	--------------------
Nitrosospira sp. C-141	--------------------
Nitrosospira tenuis Nv1	--------------------
Nitrosospira sp. Nv12	--------------------
Nitrosomonas sp. Nm63	--------------------
Nitrosomonas communis Nm2	--------------------
Nitrosomonas cryotolerans Nm55	--------------------
Nitrosomonas europaea Nm50	---N--------------N-
Nitrosomonas eutropha Nm57	--------------------
Nitrosomonas halophila Nm1	--------------------
Nitrosomonas marina Nm22	--------------------
Nitrosomonas ureae Nm10	--------------------
Nitrosococcus mobilis Nc2	--------------------
	5' **CGTAGTCCGGATCGGAGTCT** 3'
	||||||||||||||||||||
	GCATCAGGCCTAGCCTCAGA
	***βAMOr** (rev)*

Fig. 2. A comparison between oligonucleotides reported to be specific for the majority of βAOB and the available 16S rRNA/DNA sequences (> 1 kb) from these bacteria. The oligonucleotides presented in a) and b) do not cover the thirteen environmental 16S rDNA sequences (> 1 kb) reported by STEPHEN et al. (1996). The NitC probe and the NitF primer in d) have both been extended with an 'A' in position 13, according to the author's own corrections (Ward, pers. comm.). Multiple sequence alignment was performed in ClustalW (THOMPSON et al., 1994). Oligonucleotides that have been reported in a reverse direction are compared to the 16S rDNA sequences through their reversed, complementary sequence. Positions in the 16S rDNA molecule are given in *E. coli* numbering (BROSIUS et al., 1978).

2c

	258 277	632 649	632 653	638 657
Nitrosospira sp. 40KI	------.-------------	------------------	----------------------	--------------------
Nitrosospira sp. III2	------.-------------	------------------	----------------------	--------------------
Nitrosospira sp. III7	------.-------------	------------------	----------------------	--------------------
Nitrosospira sp. AF	------.-------------	------------------	----------------------	--------------------
Nitrosospira sp. AHB1	------.-------------	------------------	----------------------	--------------------
Nitrosospira sp. B6	------.-------------	------------------	----------------------	--------------------
Nitrosospira sp. C-128	------.-------------	------------------	----------------------	--------------------
Nitrosospira sp. D11	------.----C--------	------------------	----------------------	--------------------
Nitrosospira sp. GM4	------T-------------	------------------	----------------------	--------------------
Nitrosospira sp. L115	------.-------------	------------------	----------------------	--------------------
Nitrosospira sp. NpAV	------.-------------	------------------	----------------------	--------------------
Nitrosospira sp. Np22-21	------.-------------	------------------	----------------------	--------------------
Nitrosospira multiformis Nl13	------.-------------	------------------	----------------------	--------------------
Nitrosospira sp. C-141	------.-------------	------------------	----------------------	--------------------
Nitrosospira tenuis Nv1	------.-------------	------------------	----------------------	--------------------
Nitrosospira sp. Nv12	------.---------N---	----------G-------	----------G-----------	----G---------------
Nitrosomonas sp. Nm63	------.A--C---------	----------------A-	----------------------	--------------------
Nitrosomonas communis Nm2	------.-------------	-N-----N----NTNTNA	-N-----N----NTNTNA--N-	-N----NUNUNA--N-----
Nitrosomonas cryotolerans Nm55	------.----C--------	----------------A-	----------------------	--------------------
Nitrosomonas europaea Nm50	------.-------------	--N--NN-----------	--N--NN---------------	N-------------------
Nitrosomonas eutropha Nm57	------.-------------	----------------A-	----------------------	--------------------
Nitrosomonas halophila Nm1	------.-------------	--------NN--------	--------NN------------	--NN----------------
Nitrosomonas marina Nm22	------.---C---------	----------------A-	----------------------	--------------------
Nitrosomonas ureae Nm10	-----T.-------------	-----------------A	-----------------A----	-----------A--------
Nitrosococcus mobilis Nc2	------.---C---------	------------G-----	------------G---------	------G-------------
pH7B/D3 (acc. no. z69177)	------.-------------	------------------	----------------------	--------------------
pH4.2A/3F (acc. no. z69156)	------.-------------	------------------	----------------------	--------------------
pH7C/56 (acc. no. z69197)	------.-------------	------------------	----------------------	--------------------
EnrichZD5 (acc. no. z69146)	------.-------------	------------------	----------------------	--------------------
pH4.2A/3E (acc. no. z69155)	------.-------------	------------------	----------------------	--------------------
pH4.2A/H2 (acc. no. z69165)	------.-------------	--A----TG---G-----	--A----TG---G---------	-UG---G------------A
pH4.2A/G2 (acc. no. z69164)	------.-------------	------------------	----------------------	--------------------
EnvC2-23 (acc. no. z69125)	------.A------------	-----------------A	-----------------A----	-----------A----G---
EnvB1-17 (acc. no. z69104)	------.A------------	-----------------A	-----------------A----	-----------A--------
EnvA2-4 (acc. no. z69094)	------.-------------	------------------	----------------------	--------------------
pH4.2A/23 (acc. no. z69151)	-----T.---C--------T	------------G--G--	------------G--G------	------G--G--------N-
EnvA2-13 (acc. no. z69097)	------.---C---------	-----------------A	-----------------A----	-----------A--------
EnvA1-21 (acc. no. z69091)	------.---C---------	-----------------A	-----------------A----	-----------A--------
5'	**GGTAAA.GGCTTACCAAGGC**	**GCGTTTGAAACTACAAGG**	**GCGTTTGAAACTACAARGCTAG**	**GAAACUACAARGCUAGAGUG** 3'
	AAO-258 (fwd)	||||||||||||||||||	||||||||||||||||||||||	||||||||||||||||||||
		CGCAAACTTTGATGTTCC	**CGCAAACTTTGATGTTYCGATC**	**CTTTGATGTTYCGATCTCAC**
		TAO (rev)	*CTO654r (rev)*	*NITROSO4E (rev)*

2d

```
                                        738               758  844              862  846            862  1213              1233  1224             1243
Nitrosospira sp. 40KI                   ---------------------  -----------------CG-  --A------------CG-  ---------------------  --------------------
Nitrosospira sp. III2                   ---------------------  -----------------CG-  --A------------CG-  ---------------------  --------------------
Nitrosospira sp. III7                   ---------------------  ----G----------C-CG-  -------------C-CG-  ---------------------  --------------------
Nitrosospira sp. AF                     ---------------------  ---------------C----  --A----------C----  ---------------------  --------------------
Nitrosospira sp. AHB1                   ---------------------  ----G----------C----  -------------C----  ---------------------  --------------------
Nitrosospira sp. B6                     ---------------------  ----G----------C-CG-  -------------C-CG-  ---------------------  --------------------
Nitrosospira sp. C-128                  ---------------------  ----G----------C----  -------------C----  ---------------------  --------------------
Nitrosospira sp. D11                    ---------------------  -----------------CG-  --A------------CG-  ---------------------  --------------------
Nitrosospira sp. GM4                    ---------------------  -----------------CG-  --A------------CG-  ---------------------  --------------------
Nitrosospira sp. L115                   ---------------------  ---------------C-CG-  --A----------C-CG-  ---------------------  --------------------
Nitrosospira sp. NpAV                   ---------------------  ......---------C----  ....---------C----  ---------------------  --------------------
Nitrosospira sp. Np22-21                ---------------------  ......---------C----  ....---------C----  ---------------------  --------------------
Nitrosospira multiformis Nl13           ---------------------  ---------------C----  --A----------C----  ---------------------  --------------------
Nitrosospira sp. C-141                  ---------------------  ---------------C----  --A----------C----  ---------------------  --------------------
Nitrosospira tenuis Nv1                 ---------------------  ---------------C----  --A----------C----  ---------------------  --------------------
Nitrosospira sp. Nv12                   ---------------------  ----G--------GCG----  -----------GCG----  ---------------------  --------------------

Nitrosomonas sp. Nm63                   ---------------------  CTA-G---A-----------  A----A------------  ---------------------  --------------------
Nitrosomonas communis Nm2               ----A----------------  -AA--T-----------NN-  A-AT-----------NN-  ---------------------  --------------------
Nitrosomonas cryotolerans Nm55          ---------------------  NA--NT--------------  --NT--------------  ---------------------  --------------------
Nitrosomonas europaea Nm50              -------G-------------  -A---T-----------CG-  --AT-----------CG-  -----N-----N-------N-  N-------N-----------
Nitrosomonas eutropha Nm57              -------G-------------  -A---T--------------  --AT--------------  ---------------------  --------------------
Nitrosomonas halophila Nm1              -----C-G-------------  -A---T-----------CG-  --AT-----------CG-  ---------------------  --------------------
Nitrosomonas marina Nm22                ---------------------  CTA-G---A-----------  A----A------------  ---------------------  --------------------
Nitrosomonas ureae Nm10                 ----------------A----  -NN--T---------N----  N-AT---------N----  ---------------------  --------------------

Nitrosococcus mobilis Nc2               ---------------------  ----G----------C----  -------------C----  ---------------------  ------------------A-

pH7B/D3 (acc. no. z69177)               ---------------------  ---------A-----C----  --A---A------C----  ---------------------  -----------------A--
pH4.2A/3F (acc. no. z69156)             ---------------------  ---------------C----  --A----------C----  ---------------------  --------------------
pH7C/56 (acc. no. z69197)               ---------------------  ----G----------C-CG-  -------------C-CG-  ---------------------  --------------------
EnrichZD5 (acc. no. z69146)             ---------------------  ----G----------C-CG-  -------------C-CG-  ---------------------  --------------------
pH4.2A/3E (acc. no. z69155)             ----------------T----  ----G----------C----  -------------C----  ---------------------  --------------------
pH4.2A/H2 (acc. no. z69165)             ----ACCTGT---------T-  ----G----------C-CG-  -------------C-CG-  ---------------------  --------------------
pH4.2A/G2 (acc. no. z69164)             ---------------------  ----G----------C----  -------------C----  ---------------------  --------------------
EnvC2-23 (acc. no. z69125)              ------C--------------  ----G---------------  ------------------  ---------------------  --------------------
EnvB1-17 (acc. no. z69104)              ------C--------------  ----G---------------  ------------------  ---------------------  --------------------
EnvA2-4 (acc. no. z69094)               ------C--------------  ----G---------------  ------------------  ---------------------  --------------------
pH4.2A/23 (acc. no. z69151)             ---------------------  -A.-GT-----------CG-  .--T-----------CG-  ---------------------  ------------------N-
EnvA2-13 (acc. no. z69097)              ---------------------  -TA--T--------------  A-AT--------------  ---------------------  --------------------
EnvA1-21 (acc. no. z69091)              ---------------------  -TA--T--------------  A-AT--------------  ---------------------  -----------------N--

                                     5' CTGGGTTAACACTGACGCTCA  ACGGACTTGGTAACGTAGCT  GGGCTTGGTAACGTAGCT  TGGGTAGGGCTTCACACGTAA  UCACACGUAAUACAAUGGCG 3'
                                              Amβ (fwd)        ||||||||||||||||||||  ||||||||||||||||||  |||||||||||||||||||||  ||||||||||||||||||||
                                                               TGCCTGAACCATTGCATCGA  CCCGAACCATTGCATCGA  ACCCATCCCGAAGTGTGCATT  AGTGTGCATTATGTTACCGC
                                                                    NitF (rev)           NitC (rev)            NitB (rev)           Nso1225 (rev)
```

er, reveal both the benefits and the most obvious difficulties with each oligonucleotide. It should be emphasized, that only a few full length 16S rDNA sequences are known, and that the design and evaluation of the oligonucleotides positioned towards the ends of the 16S rRNA gene therefore are based on a significantly lower number of 16S rDNA sequences.

Thirteen of the thirty published oligonucleotides have been classified as broad range oligonucleotides, i. e. specific for the majority of 16S rDNA sequences from βAOB. Figure 2 shows the alignments of these primers to thirty-eight 16S rDNA-sequences from the βAOB (the 16S rDNA sequences from γAOB are not included). The remaining seventeen oligonucleotides have been reported to recognize various subclasses of βAOB; five are *Nitrosospira*-specific (Figure 3), four are specific for the whole *Nitrosomonas* cluster (Figure 4), and eight are specific for phylogenetic lines within the *Nitrosomonas* cluster (Figure 5).

The quality of the AOB-specific oligonucleotides is highly variable. Some are very promising, however, with a high degree of similarity to the 16S rDNA sequences from AOB available today, and a low degree of similarity to other bacterial 16S rDNA sequences. The specific oligonucleotides reported by MOBARRY et al. (1996 and 1997) and POMMERENING-RÖSER et al. (1996) seem presently to be the best alternatives for the detection of 16S rRNA/DNA from AOB.

The broad range oligonucleotides; specific for the majority of βAOB

MCCAIG et al. (1994) were the first to publish primers specific for the 16S rRNA genes of βAOB. These primers were denoted βAMOf and βAMOr, and are aligned to the known 16S rDNA sequences (> 1 kb) from βAOB in Figures 2a and 2b, respectively. βAMOf is identical to all of the aligned sequences, and a search in the nucleotide sequence database revealed an acceptable specificity for βAOB. A few non-AOB 16S rDNA-sequences will be recognized, however; e. g. from the soil and freshwater bacteria *Chromobacterium indigoferum* and *Spirillum volutans* (identical sequences) and the soil bacterium *Variovorax paradoxus* (one mismatch). The risk of amplifying non βAOB-16S rDNA is reduced if combining the βAOB-primer with a suitable reverse primer.

The reverse primer published by MCCAIG et al. (1993), βAMOr, is significantly less specific than the forward primer. As Figure 2b shows, βAMOr is identical to every 16S rDNA sequence from βAOB, but a search in the databases reveals that a large number of non-AOB 16S rDNA sequences also are identical to this primer [e. g. the 16S rDNA sequences from several of the widespread *Aeromonas* spp. and *Vibrio* spp. (γ-subgroup)]. Hence, the specificity of this primer is rather low, and it should not be classified as βAOB-specific. In combination with e. g. the βAMOf primer, however, the specificity level will be considerably increased. The βAMOf/βAMOr primer pair has been used recently to amplify βAOB-related 16S rDNA sequences from various environmental samples (STEPHEN et al., 1996; KOWALCHUK et al., 1997), and seems to have a good potential for this purpose.

VOYTEK and WARD (1995a and b) have published three oligonucleotides (NitA, NitB and NitC; Figures 2a and 2d), considerably more specific than the primer pair described by MCCAIG et al. (1993). The forward primer, NitA (Figure 2a), is similar to many of the known 16S rDNA sequences from βAOB, but up to five mismatches can be found, some close to the 3′ end. According to the nucleotide sequence databases, NitA has a similar specificity for the 16S rDNA from some non-AOB [e. g. from the β-subgroup soil bacteria *Spirillum voultans* (three mismatches) and *Chromobacterium indigoferum* (five mismatches)]. Using NitA under conditions in which sequences with five mismatches are recognized therefore involves a risk of amplifying false positives.

The NitB (reverse) primer (VOYTEK and WARD, 1995a) has a broader specificity than the NitA primer. It is identical to all known 16S rDNA sequences (> 1 kb) from βAOB (Figure 2d), but a database search reveals that NitB is also identical to the 16S rDNA from the soil organisms *Spirillum volutans* and *Gallionella ferruginea*, which are closely related to the βAOB. NitB has only one mismatch with the 16S rDNA sequences from a large number of non-AOB; e. g. members of the β-subgroup bacteria *Azoarcus* spp. (from sediments) and *Ralstonia solanacearum* (plant pathogen) which it probably will recognize. However, if used in combination with a forward primer without specificity for the same non-AOB 16S rDNA, NitB is probably a good choice for the detection of unknown AOB.

The oligonucleotide NitC (Figure 2d) was designed as an internal probe, to verify that products amplified using NitA and NitB in fact were βAOB-sequences (VOYTEK and WARD, 1995a and b; WARD, pers. comm.). NitC has a lower specificity for 16S rDNA sequences from βAOB than most of the other broad range oligonucleotides, and is therefore not the best alternative for βAOB identification. One should be aware, that according to the databases, the NitC-sequence has only two mismatches with the 16S rDNA sequences from two *Burkholderia* spp. (soil), and four mismatches with representatives of the *Chromobacterium* (soil and fresh water) (both β-subgroup of the Proteobacteria). NitC should not be used as a broad range PCR primer, because of mismatches in its 3′ end (Figure 2d). Recently, a variant of the NitC probe, denoted NitF, was reported (WARD et al., 1997; WARD, pers. comm.). NitF was reported to have a lower specificity for *Nitrosomonas* spp. than NitC (Figure 2d). Considering similarity to 16S rDNA sequences from non-βAOB, the NitF variant has approximately the same specificity as the NitC probe.

HIORNS et al. (1995) have reported a broad range probe named AAO258, which was designed to recognize every 16S rDNA molecule originating from *terrestrial* AOB. AAO258 is aligned to the known 16S rDNA sequences (> 1 kb) from βAOB in Figure 2c, exhibiting a close similarity with these sequences. HIORNS et al. (1995) used highly stringent conditions for hybridization

experiments with this probe, allowing only identical sequences to be recognized. Among the terrestrial 16S rDNA sequences in Figure 2c, the sequences from the two soil isolates *Nitrosospira* ssp. D11 and GM4 (UTÅKER et al., 1995), and the soil sequence pH4.2A/23 (STEPHEN et al., 1996) will probably not be recognized under these hybridization conditions (one and three mismatches, respectively). The 16S rDNA from the other terrestrial βAOB, including some of the non-terrestrial ones, will be recognized by this probe, however. Unfortunately, a search with the AAO258 sequence in the databases reveals that it is identical to a number of 16S rDNA sequences from non-AOB, ranging from members of the closely related β-subgroup bacterium *Azoarcus* (from sediments) to the distantly related Gram positive genus *Lactobacillus*. This fact limits the use of this probe.

MOBARRY et al. (1996 and 1997) have published two broad range probes, called Nso190 and Nso1225 (Figures 2a and 2d, respectively). The Nso190 probe is identical to all known *Nitrosospira*-16S rRNA (> 1 kb), but has a few mispairings to some of the *Nitrosomonas*-sequences. The conditions for the use of this probe were adjusted to recognize all 16S rRNA sequences which had less than two mismatches (MOBARRY et al., 1996), which seems sufficient for recognizing all 16S rRNA sequences originating from βAOB, except the *Nitrosomonas ureae* Nm10 sequence (three mismatches). This indicates that there may be unknown βAOB-16S rRNA sequences that do not match with the Nso190 probe. Lowering the stringency, to obtain hybridization to *Nitrosomonas ureae* Nm10, is not advisable, because there exists non-AOB 16S rRNA sequences with two and three mismatches with the Nso190 probe [e. g. 16S rRNA from the soil bacterium *Variovorax paradoxis* and the fresh water bacterium *Rhodocyclus tenuis* (both β-subgroup)]. The complementary sequence of Nso190 should be well suited as a forward PCR primer, however, as has been shown by KOWALCHUK et al. (1997). Nso190 is also a good choice in a control hybridization after specific PCR amplification, using lower stringency conditions.

The other broad range probe reported by MOBARRY et al. (1996 and 1997), designated Nso1225, is presented in Figure 2d. This probe is partly overlapping with the NitB primer (VOYTEK and WARD, 1995b), but has a better specificity. Using the hybridization conditions recommended by MOBARRY et al. (1996), probe Nso1225 will recognize 16S rRNAs with less than two mismatches. Hence, every 16S rRNA sequence in Figure 2d will hybridize to this probe. A search with the Nso1225 sequence in the databases, shows that under the suggested hybridization conditions this probe will only recognize the 16S rRNA molecules originating from the βAOB [the closely related fresh water bacterium *Gallionella ferruginea* is the only exception, also mentioned by the authors (MOBARRY et al., 1996)]. Therefore, Nso1225 is a good choice for the detection of βAOB in an environmental sample, and it can apparently also be used as a reverse primer in a PCR reaction.

HOVANEC and DELONG (1996) have published the broad range probe NITROSO4E, supposed to recognize all βAOB organisms (Figure 2c). Under the recommended hybridization conditions for this probe, only identical sequences were recognized, which probably is too strigent: most of the 16S rRNA sequences from βAOB have less than two mismatches with this probe, but up to four mismatches can be found [the pH4.2A/H2-sequence reported by STEPHEN et al. (1996)]. Lowering the stringency conditions is not adivsable, however, because NITROSO4E is identical, or has one or two mispairings with several non-AOB 16S rRNA sequences; e. g. representatives of the cyanobacterial genera *Aphanizomenon* and *Synechococcus*. Hence, the NITROSO4E probe has a biased specificity for the βAOB.

Recently, a new primer specific for the *terrestrial* AOB was published; TAO_{rev}, presented in Figure 2c (CHANDLER et al., 1997). This primer is parly overlapping with the NITROSO4E primer (HOVANEC and DELONG, 1996), but has a slightly improved specificity. Again, the pH4.2A/H2-sequence (STEPHEN et al., 1996) will not be recognized, but every terrestrial AOB, and also the majority of the non-terrestrial βAOB will be detected by this primer. Searching the databases with the TAO_{rev} sequence reveals that it is identical to the 16S rDNA from a few methane-oxidizing bacteria in addition to the βAOB, and has one or two mismatches to a few other non-AOB 16S rDNA sequences. The use of TAO_{rev} therefore involves a risk of loosing some unknown 16S rDNA sequences from βAOB, and at the same time a risk of amplifying 16S rDNA from non-βAOB organisms.

CHANDLER et al. (1997) did also report a forward primer (TAO_{fwd}, not shown), the nucleotide sequence of which is contained within the Nso190 sequence, already published by MOBARRY et al. (1996). According to the sequence databases, the TAO_{fwd} primer has a specificity comparable to the probe Nso190, although Nso190, being longer, seems to be somewhat more specific.

KOWALCHUK et al. (1997) did in a recent study design four new PCR primers specific for the βAOB, being improvements of some of the oligonucleotide sequences already published. The forward primers in this study, collectively referred to as CTO189f-GC, were linked to a GC-clamp, for use in a denaturing gradient gel electrophoresis (DGGE) analysis. CTO189f-GC (Figure 2a) was a mixture of three oligonucleotide sequences, complementary to the probe Nso190 reported by MOBARRY et al. (1996). The combination of variable positions in the mixed forward primers gives an increased specificity for βAOB, compared to Nso190, but the specificity for non-AOB is the same as already reported (MOBARRY et al., 1996). The "mixed primer" CTO189f-GC, is identical to all 16S rDNA sequence from βAOB, except for one mismatch with two *Nitrosomonas*-sequences. Since the mismatches are not in the 3′ end, these 16S rDNA sequences will still be recognized (KOWALCHUK et al., 1997).

KOWALCHUK et al. (1997) also presented a reverse primer, called CTO654r (Figure 2c), overlapping with NITROSO4E (HOVANEC and DELONG, 1996) and TAO_{rev} (CHANDLER et al., 1997). CTO654r has an improved specificity compared to the overlapping oligonucleotides,

with less than two mismatches with every 16S rDNA sequence from βAOB, except for two of the environmental sequences reported by STEPHEN et al. (1996). Again, the exclusion of known 16S rDNA sequences indicates that this primer may fail to recognize other, unknown 16S rDNA sequences from βAOB. According to the databases, CTO654r is identical to 16S rDNA sequences from βAOB only, but it has only one mismatch with the 16S rDNA from some methane-oxidizing bacteria (γ-subgroup), which will be recognized. In combination with the CTO189f-primers, however, the specificity for βAOB will be satisfying.

Oligonucleotides reported to recognize members of the *Nitrosospira* genus only (including the basonymes *Nitrosolobus* and *Nitrosovibrio*)

HIORNS et al. (1995) were the first to publish primers supposed to be specific for the members of the *Nitrosospira* genus only. Their forward primer, NS-85, is aligned to the known 16S rDNA sequences (> 1 kb) from *Nitrosospira* spp. in Figure 3, showing a high degree of similarity. According to the databases, the NS-85 primer has a low degree of similarity to the other genera of AOB. Unfortunately, however, this primer is identical to the 16S rDNA sequences from a number of other bacteria [e. g. members of the soil/water/sewage genus *Acinetobacter* (γ-subgroup)] and can consequently not be categorized as *Nitrosospira*-specific. Obviously, if used for PCR, the specificity of NS-85 can be improved by a suitable reverse primer.

The reverse primer in the same report, called NS-1009 (Figure 3), was also designed to amplify *Nitrosospira* spp. only (HIORNS et al., 1995). NS-1009 has a narrower specificity than the NS-85 primer, but has up to six mismatches with the aligned *Nitrosospira*-sequences in Figure 3. Some of the exhibited mismatches are even in the 3′ end of the primer, and NS-1009 will clearly fail to recognize these sequences. Consequently, the NS-1009 may fail to recognize the 16S rDNA from other, unknown *Nitrosospira* spp. Comparing the NS-1009 sequence with the nucleotide sequence databases reveals that it will recognize only a few 16S rDNA sequences from non-*Nitrosospira* organisms (phytopathogenic pseudomonads), and that it has a very low similarity to the 16S rDNA from *Nitrosomonas* spp. The NS-1009 primer is perhaps better suited as a specific probe, under low stringency conditions (HASTINGS et al., 1997).

MOBARRY et al. (1996) have designed a *Nitrosospira* specific probe called Nsv443, shown in Figure 3. This probe has a good similaritrity to the 16S rRNA from the *Nitrosospira* subclass of the AOB, and a correspondingly low similarity to other AOB-16S rRNA sequences. If using the suggested hybridization conditions, the Nsv443 probe will bind to 16S rRNA sequences with less than three mismatches (MOBARRY et al., 1996). As is shown in Figure 3, every known 16S rRNA sequence from *Nitrosospira* spp., except for the isolate D11-sequence (three mismatches), will be recognized under these conditions. According to a sequence database search, the Nsv443 will also recognize a few, unknown bacterial 16S rRNA sequences, but all other non-AOB organisms and every *Nitrosomonas* spp. have 16S rRNAs with low similarity to Nsv443. Hence, it should be possible to lower the stringency when using Nsv443, in order to detect all known 16S rRNA gene sequences from *Nitrosospira* (also the D11-sequence), and still have an acceptable specificity. The Nsv443 oligonucleotide will probably also be a suitable PCR primer, as none of its mismatches with the *Nitrosospira* 16S rRNA sequences are in the 3′ end.

POMMMERENING-RÖSER et al. (1996) have published the *Nitrosospira*-specific primer designated Nsp0 (Figure 3). Primer Nsp0 is partly overlapping with the Nsv443 primer, but is less reliable, since it has mismatches with some *Nitrosospira* 16S-sequences close to its 3′ end. A database search with this primer reveals that it is identical to 16S rDNA sequences from *Nitrosospira* spp. only: except for one *Salmonella*-sequence, it has more than two mismatches with every non-*Nitrosospira* sequence. Nsp0 has a low similarity to *Nitrosomonas*-sequences.

Recently, there was a study by HASTINGS et al. (1997), presenting a new probe, Nlm459r (Figure 3), made on the basis of the 16S rDNA sequences from *Nitrosospira multiformis* N113^T and *Nitrosospira* sp. C-141. This probe is partly overlapping with the Nsp0-primer (POMMERENING-RÖSER et al., 1996), but has a narrower specificity. Nlm459r is identical to ten 16S rDNA sequences (>1 kb) from *Nitrosospira*, but has up to five mismatches with other *Nitrosospira*-sequences (Figure 3). If using the hybridization conditions recommended by HASTINGS et al. (1997), only identical sequences will be recognized. Judged from a database search with the Nlm459r-sequence, it seems possible to lower the stringency to recognize sequences with less than four mismatches, to get a broader specificity for *Nitrosospira*-16S rDNA sequences. A few 16S rDNA sequences from *Nitrosospira* spp. would still not be recognized, however. The Nlm459r probe exhibits low similarity to 16S rDNA from *Nitrosomonas* spp.

Oligonucleotides reported to recognize the *Nitrosomonas* cluster only

The *Nitrosomonas* cluster is a monophyletic group containing all known species of *Nitrosomonas* together with *Nitrosococcus mobilis* (Figure 1). Because *N. mobilis* is so closely related to *Nitrosomonas* spp., it has been proposed that this species should be transferred to the *Nitrosomonas* genus (HEAD et al., 1993; WAGNER et al., 1995).

HIORNS et al. (1995) have published the primers NM-75 and RNM-1007, aligned to the known 16S rDNA sequences (> 1 kb) from the *Nitrosomonas*-cluster in Figures 4a and b, respectively. These primers were designed when only three 16S rDNA sequences from *Nitrosomonas* were known, and they were characterized as specific for the non-marine *Nitrosomonas* spp. at the time. Today, the number of available 16S rDNA se-

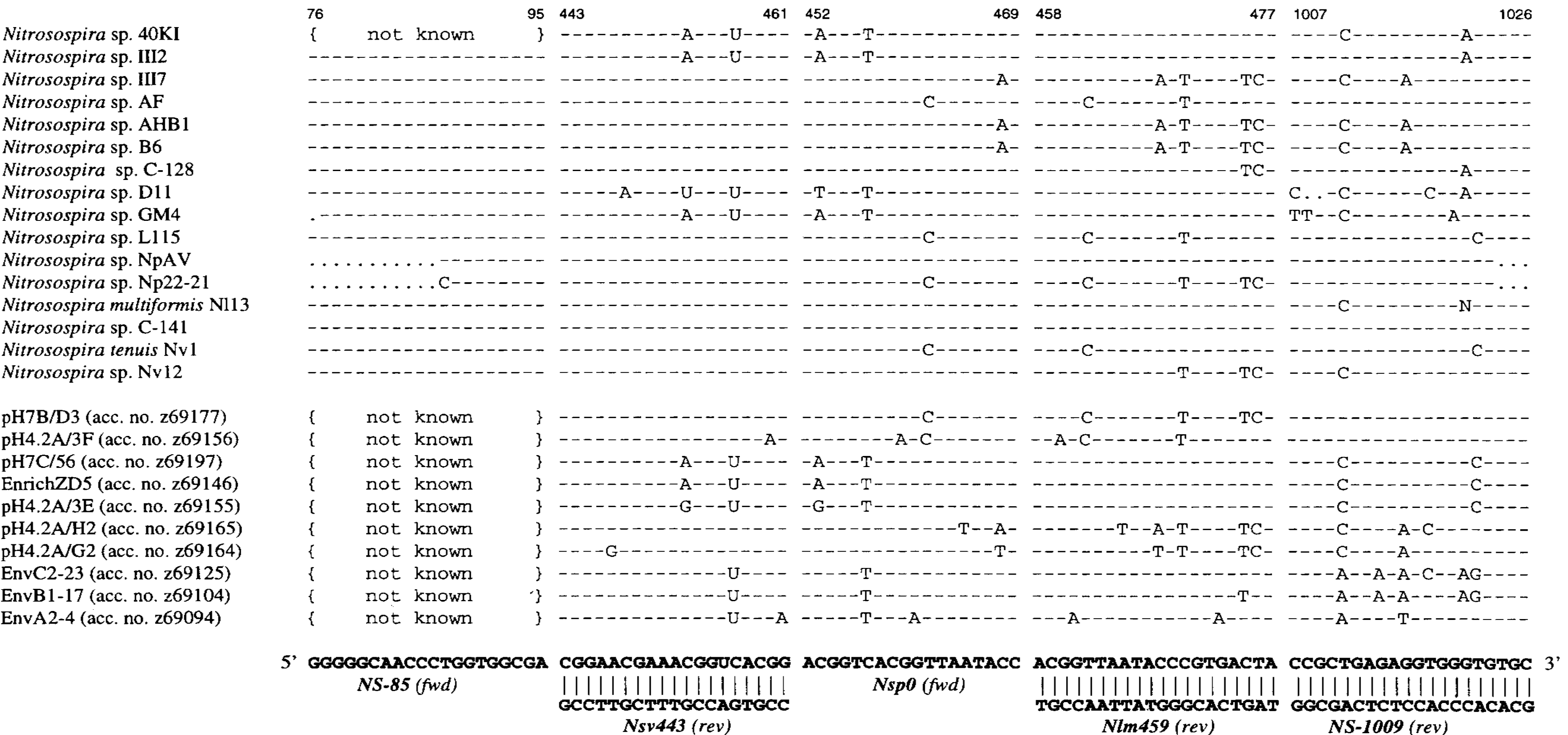

Fig. 3. A comparison between oligonucleotides reported to be specific for the *Nitrosospira* genus and the available 16S rRNA/DNA sequences (> 1 kb) from this genus. Ten of the environmental 16S rDNA sequences (> 1 kb) reported by Stephen et al. (1996) are closely related to the *Nitrosospira* 16S rDNA sequences, and are hence a part of the comparison. The NS-85 primer does not cover these environmental sequences. Multiple sequence alignment was performed in ClustalW (Thompson et al., 1994). Oligonucleotides that have been reported in a reverse direction are compared to the 16S rDNA sequences through its reversed, complementary sequence. Positions in the 16S rDNA molecule are given in *E. coli* numbering (Brosius et al., 1978).

a)

```
                                   67                 86  148             165  155               173
Nitrosomonas sp. Nm63              -------AC---G-G-TTG-   ------------------   -------------------
Nitrosomonas communis N. 12        {     not known    }   ------------------   -------------------
Nitrosomonas cryotolerans Nm55     {     not known    }   ----------------N-   ---------N---------
Nitrosomonas europaea Nm50         --------------------   ------------------   -------------NN----
Nitrosomonas eutropha Nm57         --------------------   ------------------   -----------NNNN----
Nitrosomonas halophila Nm1         {     not known    }   ------------------   -----------N-------
Nitrosomonas marina Nm22           {     not known    }   ------------------   -------------------
Nitrosomonas ureae Nm10            {     not known    }   ------------------   -------------------

Nitrosococcus mobilis Nc2          {     not known    }   ------NNNNN-------   NNNN--  -----------

                                5' CGGCAGCGGGGGCTTCGGCC   ATAACGCATCGAAAGATG   AUCGAAAGAUGUGCUAAUA 3'
                                       NM-75 (fwd)            Nm0 (fwd)        |||||||||||||||||||
                                                                               TAGCTTTCTACACGATTAT
                                                                                   Nsm156 (rev)
```

b)

```
                                   1005                  1028
Nitrosomonas sp. Nm63              -T-TC-A----T-G-TT---G--TT
Nitrosomonas communis Nm2          -TCC-CA----TG-GGA---G----
Nitrosomonas cryotolerans Nm55     G--CGCT----GG-G-----G----
Nitrosomonas europaea Nm50         -------------------------
Nitrosomonas eutropha Nm57         -------.---------NN--N----
Nitrosomonas halophila Nm1         --CTG---------NN----N----
Nitrosomonas marina Nm22           -T-TC-A----T-G-TT---G--NN
Nitrosomonas ureae Nm10            -N-TNCA----TGN-NNT--G-N--

Nitrosococcus mobilis Nc2          -T-T-C----------A---G----

pH4.2A/23 (acc. no. z69151)        GT-TT-A----T-A-TTC--G----
EnvA2-13 (acc. no. z69097)         -A-TT-A----T-AG-T---G--TT
EnvA1-21 (acc. no. z69091)         -A-TT-A----T-G--T---G--TT

                                5' TCTAATGGAGACATAAGAGTACCCG 3'
                                   ||||||||||||||||||||||||
                                   AGATTACCTCTGTATTCTCATGGGC
                                        RNM-1007 (rev)
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Fig. 4. A comparison between oligonucleotides reported to be specific for the *Nitrosomonas* cluster (including *Nitrosococcus mobilis*) and the available 16S rRNA/DNA sequences (> 1 kb) from this cluster. Three of the environmental 16S rDNA sequences (> 1 kb) reported by STEPHEN et al. (1996) are closely related to the *Nitrosomonas* cluster, and are therefore a part of the comparison with primer RNM-1007 (b), which is the only oligonucleotide in this category that covers these sequences. Multiple sequence alignment was performed in ClustalW (THOMPSON et al., 1994). Oligonucleotides that have been reported in a reverse direction are compared to the 16S rDNA sequences through their reversed, complementary sequence. Positions in the 16S rDNA molecule are given in *E. coli* numbering (BROSIUS et al., 1978).

quences from *Nitrosomonas* has increased, and neither NM-75 nor RNM-1007 recognize the new sequences from non-marine origins (POMMERENING-RÖSER et al., 1996). Also, when searching the databases with these two primer sequences, one can see that the NM-75 primer is identical to several non-AOB 16S rDNA sequences [e. g. the closely related β-subgroup bacterium *Azoarcus* (from sediments)] while RNM-1007 has a low degree of similarity to 16S rDNA sequences in general. It can not be recommended to use the NM-75/RNM-1007-primer pair for a general detection of non-marine *Nitrosomonas* spp. today, as this primer pair seems to be specific for *N. europaea* and *N. eutropha* only.

The probe Nsm 156, published by MOBARRY et al. (1996), has been reported to recognize the whole *Nitrosomonas*-cluster. As Figure 4a shows, this probe is indeed identical to the known *Nitrosomonas*-16S rRNA sequences, and to the *Nitrosococcus mobilis* Nc2 16S rRNA. A search in the databases with the Nsm156 probe sequence shows that it has one or two mismatches to a number of other 16S rRNA sequences, both from other βAOB and from other bacteria. However, under the high stringency conditions recommended by MOBARRY et al. (1996), only identical sequences will be recognized, and Nsm156 is therefore a good choice for the detection of members of the *Nitrosomonas*-cluster.

POMMERENING-RÖSER et al. (1996) have published several *Nitrosomonas*-specific primers, of which one, the Nm0 (Figure 4a), was assumed to be specific for the whole *Nitrosomonas*-cluster. However, this primer is identical to seven of the *Nitrosospira*-16S rDNA sequences as well, and therefore has too low specificity to be called *Nitrosomonas*-specific. According to the databases, Nm0 is identical to 16S rDNA from βAOB only, but has one, two or three mismatches to a number of other non-AOB sequences [e. g. from the soil and fresh water bacteria *Chromobacterium indigoferum* and *Spirillum volutans* (both β-subgroup)].

Oligonucleotides reported to recognize subgroups of the *Nitrosomonas* cluster

WAGNER et al. (1995) published the first probe in this category, designated NEU (Figure 5b), which was designed to cover most halophilic and halotolerant members of the genus *Nitrosomonas*. The NEU-probe is identical to the 16S rRNA genes from *Nitrosomonas europaea* Nm50, *N. eutropha* Nm57 and *N. halophila* Nm1, but has one or more mismatches with the other 16S rRNA-sequences from βAOB. Under the hybridization conditions used by WAGNER et al. (1995), 16S rRNA-sequences with one mismatch did also bind to NEU, thereby including *Nitrosococcus mobilis* Nc2 and six *Nitrosospira* spp. in the group of organisms recognized by this probe. The NEU probe was reported to have only one mismatch with some non-AOB 16S rRNA sequences (e. g. from the β-subgroup soil bacterium *Comamonas testosteroni*), but these sequences were successfully prevented from binding to the NEU probe by the use of competitor oligonucleotide probes (WAGNER et al., 1995). The 16S rRNA sequences that deviate with more than one nt from the NEU sequence, including members of both *Nitrosomonas* and *Nitrosospira*, was reported not to be recognized by this probe under the given hybridization conditions.

HOVANEC and DELONG (1996) have presented a probe named NSM1B, designed to recognize the 16S rRNA in

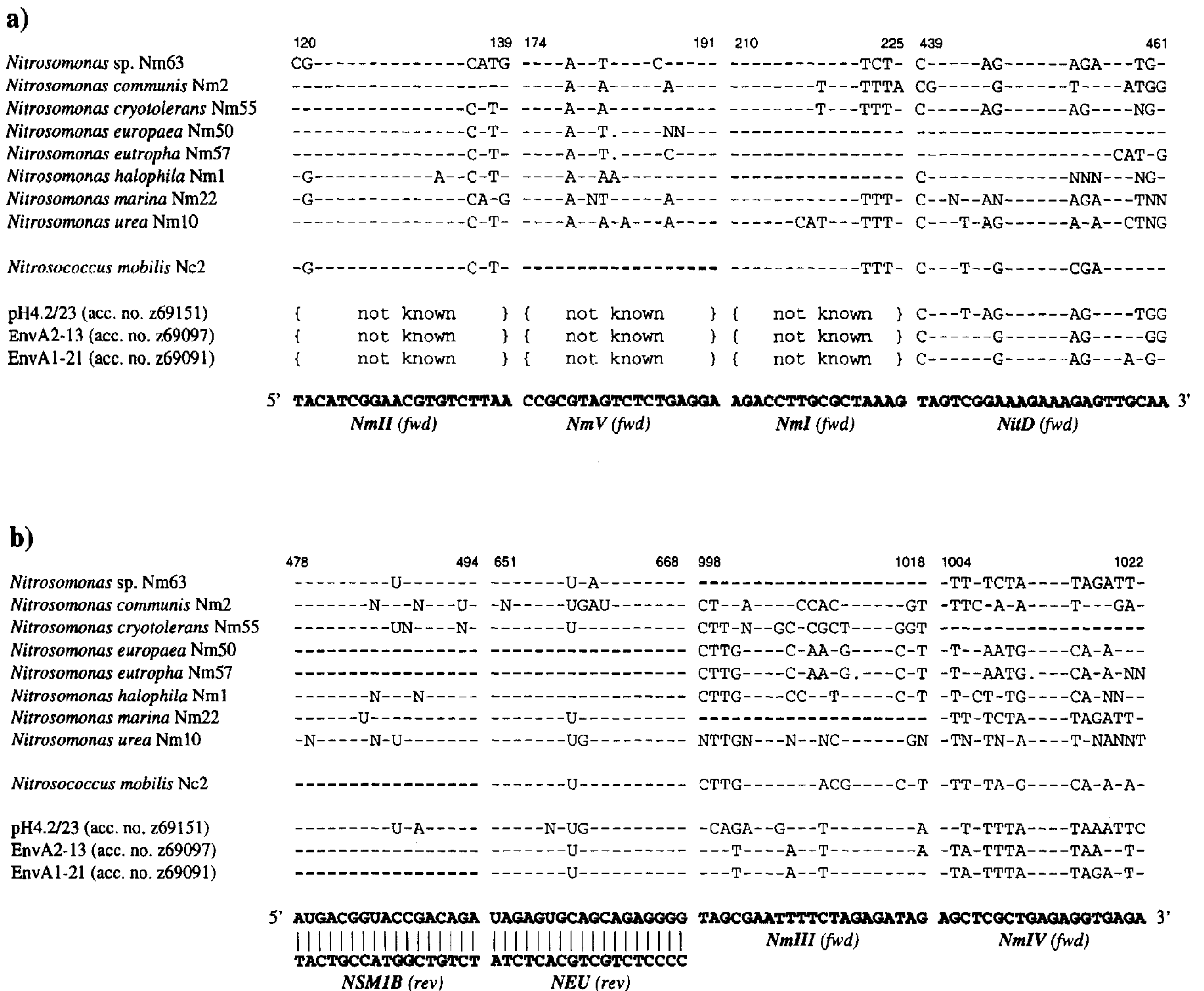

Fig. 5. A comparison between oligonucleotides reported to be specific for subgroups in the *Nitrosomonas* cluster (including *Nitrosococcus mobilis*) and the available 16S rRNA/DNA sequences (> 1 kb) from this cluster. Three of the environmental 16S rDNA sequences (> 1 kb) reported by STEPHEN et al. (1996) are closely related to the *Nitrosomonas* cluster, and are therefore a part of the comparison. Bold sequences: 16S rDNA sequences identical to the oligonucleotide. Multiple sequence alignment was performed in ClustalW (THOMPSON et al., 1994). Oligonucleotides that have been reported in a reverse direction are compared to the 16S rDNA sequences through its reversed, complementary sequence. Positions in the 16S rDNA molecule are given in *E. coli* numbering (BROSIUS et al., 1978).

the three species *Nitrosomonas europaea, N. eutropha* and *Nitrosococcus mobilis* (Figure 5d). NSM1B is identical to the 16S rRNA gene from these three species, as well as from *Nitrosomonas halophila* and two of the environmental sequences reported by STEPHEN et al. (1996). *N. halophila* belongs to the same lineage as *N. europaea* and *N. eutropha* (Figure 1), and one would expect the NSM1B probe to recognize this organism. Conversely, the similarity to the 16S rDNA sequences reported by STEPHEN et al. (1996) is somewhat surprising, since these sequences apparently are more distantly related (Figure 1). NSM1B might therefore have a broader specificity than one first thought. A search in the nucleotide sequence databases with NSM1B shows that it has at least two mispairings with every 16S rRNA molecule from *Nitrosospira* spp. It was reported, also that there are several non-AOB 16S rRNA sequences with only one mismatch with this probe sequence (HOVANEC and DELONG, 1996). Hence, the NSM1B probe must be used with very high stringency conditions to be specific.

In the study by POMMERENING-RÖSER et al. (1996) five new, nearly full length 16S rDNA sequences from *Nitrosomonas* spp. have been published. These sequences, in combination with some 16S rDNA partly sequences from *Nitrosomonas* spp., did make a basis for the design of four new primers specific for different subgroups within the *Nitrosomonas* cluster (NmI, NmII, NmIII and NmIV in Figure 5a and b). These primers appear all to be highly specific, both compared to an alignment of the available βAOB 16S rDNA sequences, and according to the nucleotide sequence databases. All primers are identical to their target sequences only, and can therefore be used with high stringency conditions to ensure good specificity.

POMMERENING-RÖSER et al. (1996) did also design a primer specific for the 16S rDNA from *Nitrosococcus*

Table 2. The result of a qualitative evaluation of the thirty-one different oligonucleotides available for the detection of AOB in the β-subgroup of the *Proteobacteria* today. Oligonucleotides have been graded, according to their specificity for the various subgroups under conditions recommended by the autohrs: +++ high similarity; ++ medium similarity; + low similarity; – not specific. In addition, the similarity to 16S rDNA from other organisms than AOB is stated. The grading os of course not precise, and the reader is advised to refer to the text for a detailed evaluation.

Oligo-nucleotide	*β-subgroup AOB*	*Nitrosospira spp.*	*Nitrosomonas cluster*	*Nitrosomonas subgroups*	*non AOB*
NitA	+(+)	+(+)	++		+(+)
βAMOf	+++	+++	+++		+(+)
Nso190	++(+)	+++	++(+)		+
CTO189f	+++	+++	++(+)		+
AAO258	++(+)	++(+)	++(+)		+++
TAOrev	++(+)	+++	++		++
CTO654r	++(+)	+++	++(+)		+(+)
NITRROSO4E	++	+++	++		++
Amβ	++(+)	++(+)	++(+)		+(+)
NitF	+	++	(+)		+
NitC	+(+)	+(+)	+		+(+)
NitB	+++	+++	+++		++
Nso1225	+++	+++	+++		(+)
βAMOr	+++	+++	+++		+++
NS-85		+++	–		+++
Nsv443		++(+)	–		(+)
Nsp0		++	–		(+)
Nlm459r		+(+)	–		(+)
NS-1009		+(+)	–		+
NM-75		–	+		+++
Nm0		++	+++		++
Nsm156		(+)	+++		+(+)
RNM-1007		–	+		–
NmlI		–	(–)	+++	–
NmV		–	(–)	+++	–
Nml		–	(–)	+++	–
NitD		–	(–)	+++	–
NSMIB		–	(+)	+++	+(+)
NEU		+(+)	(++)	+++	+
NmlII		–	(–)	+++	–
NmlV		–	(–)	+++	–

mobilis Nc2 only (NmV, Figure 5b). A database search with this primer reveals that it is in fact identical to the 16S rRNA gene from *Nitrosococcus mobilis* Nc2 only, and it is sufficiently different from all other known 16S rDNA sequences.

Recently, WARD et al. (1997) reported a forward primer denoted NitD (Figure 5a), amplifying 16S rDNA from *Nitrosomonas europaea* only. NitD is partly overlapping with the *Nitrosospira*-specific oligonucleotides Nsv443 (MOBARRY et al., 1996) and Nsp0 (POMMERENING-RÖSER et al., 1996), but has a nucleotide sequence identical to the *N. europaea* Nm50-sequence only. According to the databases, *N. europaea* is in fact the only organism which will be recognized by this oligonucleotide.

Conclusion

A relatively high number of oligonucleotides specific for the 16S rRNA genes in βAOB have now been published, providing a powerful tool for the detection of this type of bacteria in the environment. However, the reported specificity of many of these oligonucleotides has changed, as the number of 16S rRNA gene sequences has increased.

A summary of the present evaluation of the available specific oligonucleotides is presented in Table 2. The table is meant as a guide to select specific oligonucleotides for βAOB detection. Which oligonucleotide(s) to choose is dependent on the type of study one wants to perform: highly specific oligonucleotides are best suited for qualitative, directed studies, while quantitative studies will be least biased by the use of broad specificity oligonucleotides. Because of the limited knowledge about the total number and diversity of the natural population of ammonia-oxidizers, qualitative studies will in most cases be more conclusive. It is conceivable that with the present oligonucleotides one is still able to detect only a fraction of the actual number of AOB.

Today, it is also possible to detect AOB by the use of oligonucleotides specific for other gene sequences than the 16S rRNA/DNA, for instance the genes encoding the AOB specific enzymes ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) (ROTTHAUWE et al., 1995). Presently, the number of sequenced *amo*- and *hao*-genes is considerably lower than the number of available 16S rRNA genes, but there is a great potential in using these genes for oligonucleotide design: In the case of *amoA*, one has found a high degree of similarity between nucleotide sequences from distantly related AOB: the species *Nitrosomonas europaea* (β-subgroup) and *Nitrosococcus oceani* (γ-subgroup) have very similar *amoA*-sequences, and the gene can be amplified by the same, specific primers (SINIGALLIANO et al., 1995). A parallel observation has been done for the *Nitrosospira multiformis* N113^T (basonyme *Nitrosolobus* and *Nitrosopira* sp. strain NpAV (ROTTHAUWE et al., 1995). Despite this sequence similarity, the *amoA* genes are still less conserved than the 16S rRNA genes, making it easier to distinguish between closely related species (ROTTHAUWE et al., 1995). *amoA* specific primers have already been used successfully in the detection of unknown ammonia-oxidizing bacteria in sea water samples (SINIGALLIANO et al., 1995) and in soil samples (HASTING et al., 1997).

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