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Evaluation of PCR Primer Selectivity and Phylogenetic Specificity by Using Amplification of 16S rRNA Genes from Betaproteobacterial Ammonia-Oxidizing Bacteria in Environmental Samples▽†

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The effect of primer specificity for studying the diversity of ammonia-oxidizing betaproteobacteria (βAOB) was evaluated. βAOB represent a group of phylogenetically related organisms for which the 16S rRNA gene approach is especially suitable. We used experimental comparisons of primer performance with water samples, together with an in silico analysis of published sequences and a literature review of clone libraries made with four specific PCR primers for the βAOB 16S rRNA gene. With four aquatic samples, the primers NitA/NitB produced the highest frequency of ammonia-oxidizing-bacterium-like sequences compared to clone libraries with products amplified with the primer combinations βAMOf/βAMOr, βAMOf/Nso1255g, and NitA/Nso1225g. Both the experimental examination of ammonia-oxidizing-bacterium-specific 16S rRNA gene primers and the literature search showed that neither specificity nor sensitivity of primer combinations can be evaluated reliably only by sequence comparison. Apparently, the combination of sequence comparison and experimental data is the best approach to detect possible biases of PCR primers. Although this study focused on βAOB, the results presented here more generally exemplify the importance of primer selection and potential primer bias when analyzing microbial communities in environmental samples.

Microbial ecology has undergone a profound change in the last two decades in terms of methods employed for the analysis of natural communities. Emphasis has shifted from culturing to the analysis of signature molecules, in particular specific gene sequences. This approach often relies on amplification of target sequences by use of the PCR (32). The outcome of a PCR can be affected by factors as diverse as the biases associated with cell lysis and nucleic acid extraction, the PCR conditions, the abundance of the target sequence, and the choice of primers. Differences in the specificity (rejection of nontarget organisms) and sensitivity (discrimination of target organisms) of the primers have an effect on the detection of specific groups of microorganisms in environmental samples. Consequently, the selection of the appropriate primers for PCR is important for the outcome of these studies. Although primer sensitivity and specificity can be partially studied by in silico approaches, ultimately experimental evaluation is essential to validate the performance of the different primer pairs for PCR.

In order to analyze the influence of primer specificity and sensitivity on diversity studies, it is necessary to select a group of microorganisms for which enough information has been compiled. Ammonia-oxidizing bacteria (AOB) represent one of the bacterial groups for which the 16S rRNA gene approach has been successfully used (4, 17, 23, 30, 37, 48). AOB and the recently discovered ammonia-oxidizing archaea are autotrophic microorganisms that carry out the first step in nitrification (19, 27, 46). AOB are divided into two monophyletic groups based on their 16S rRNA gene sequences (14, 38, 39, 45). The first group belongs to the betaproteobacteria (βAOB) and includes clusters of *Nitrosomonas* (including *Nitrosococcus mobilis*) and *Nitrosospira* (including *Nitrosolobus* and *Nitrosovibrio*) species. The second group, affiliated with the gammaproteobacteria, contains *Nitrosococcus oceani* and *Nitrosococcus halophilus*. Due to their phylogenetic coherence, several 16S rRNA gene primers or probes for the specific detection of AOB by PCR or fluorescence in situ hybridization have been published (13, 15, 24, 30, 31, 36, 44, 47, 48, 50, 52). Some of these primers and probes were designed at the beginning of the molecular era, when only a limited number of sequences were available (30, 48, 49).

Our picture from AOB communities in different habitats is probably incomplete and biased by the possible limitations of PCR methods (49). Therefore, comparative analyses of the specificity and sensitivity of PCR with different primer combinations is required to interpret the results from diversity studies and to select appropriate PCR conditions for best recovery of a broad range of different nitrifying bacteria. In the case of AOB, little information is available about the influence of primer specificity and sensitivity on the outcome of diversity studies (29). In previous studies, primer bias has been evaluated mainly by comparing nucleotide sequences of the primers with known sequences of target organisms (21, 38, 47). Recently biases of several PCR strategies for studying AOB have

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TABLE 1. Primers analyzed in this study

Primer	Sequence (5′–3′)	Positions	Reference
NitA	CTT AAG TGG GGA ATA ACG CAT CG	136–158	48
NitB	TTA CGT GTG AAG CCC TAC CCA	1213–1233	48
CTO189fa	GGA GRA AAG CAG GGG ATC G	189–207	24
CTO189fb	GGA GGA AAG TAG GGG ATC G	189–207	24
CTO654r	CTA GCY TTG TAG TTT CAA ACG C	632–653	24
βAMOf	TGG GGR ATA ACG CAY CGA AAG	142–162	30
βAMOr	AGA CTC CGA TCC GGA CTA CG	1295–1314	30
Nso1225g	CGC CAT TGT ATT ACG TGT G	1224–1243	Modified from 31

been shown in experiments with denaturing gradient gel electrophoresis (DGGE) in two environmental samples (29). The aim of the present work was to study the effect of primer specificity on diversity studies of AOB. For this, we evaluated experimentally the specificity of five PCR primers designed for betaproteobacterial ammonia-oxidizing bacteria (βAMO) by comparing clone libraries generated from four aquatic environments. The analysis of clone libraries was chosen because it provides higher resolution than fragment analysis (separated by DGGE) and makes it possible to easily recognize changes of the major clades amplified with different primer combinations.

In addition, we included the analysis of published AOB clone libraries and the sequence match of the primers used to achieve a more comprehensive estimation of the suitability of different primer pairs for community analysis in a wider spectrum of habitats.

Comparison of 16S rRNA gene clone libraries from four aquatic environments. We selected four different aquatic habitats to compare the specificity of primers for amplifying the 16S rRNA gene in βAOB. The study sites included Lake Kinneret, situated in the northern part of Israel, Lake Plußsee, located in Schleswig-Holstein in Germany, and Boknis Eck, out of the Kiel Fjord in the German Baltic Sea. The sample from Lake Kinneret was collected at the central lake station (station A) during the stable stratification period at a 17.7-m depth in the metalimnetic layer, where thermocline and chemocline coincided. This depth was selected because it corresponded to the interface between oxic and anoxic water, in which nitrification is most likely to occur. From Lake Plußsee and the Baltic Sea, samples were collected from the oxic-anoxic sediment-water interface. An additional sample was collected from the water column of the Baltic Sea (20 m), which corresponds to a suboxic layer. A more detailed description of the samples from Lake Plußsee and the Baltic Sea and the DNA extraction techniques has been published previously (18).

For PCR, a nested approach was chosen to increase the detection limit of AOB, as has been suggested earlier (48). The 16S rRNA gene was amplified with the bacterial primers Eub9_27/Eub1542 (5), using the proofreading *Pfu* DNA polymerase (Promega) according to a previously described method (18). These PCR products were cleaned, diluted 100 times, and used as templates in a nested PCR with the specific βAOB primers. Nested amplification was also carried out with *Pfu* DNA polymerase (Promega) and consisted of the following: initial denaturation at 95°C for 2 min; and hot start at 80°C and 25 cycles of 95°C for 30 s, 57°C for 30 s, and 73°C for 3 min. The following primer combinations specific for βAOB were used: NitA/NitB (48), βAMOf/βAMOr (30), βAMOf/Nso1225g, and NitA/Nso1225g (Table 1). The primer Nso1225g is a modification of the probe Nso1225 (31), which lacks the final A at the 3′ end, thus facilitating primer extension by the DNA polymerase. For cloning, products of three different amplifications were pooled and cloned using the Zero Blunt PCR cloning kit (Invitrogen). From each cloning, 48 to 96 clones were picked and screened as described earlier (18). Sequences were compared with all GenBank entries using BLAST (2). Groups of unique sequences were defined according to the hits in BLAST. The groups were confirmed by alignment of the sequences with ClustalX and with the phylogenetic software program ARB (28). Although the number of clones sequenced might not describe the whole community present in the sample, it allows recognizing changes of the major clades amplified with different primer combinations.

The percentages of AOB-like sequences detected in the clone libraries were different and depended on the primer pairs used (Table 2). In all the samples, the proportion of AOB-like sequences was higher in the libraries prepared with products of the primer combination NitA/NitB. In the sample from the metalimnetic layer of Lake Kinneret, sequences from the 4 clone libraries were related to 10 different groups of bacteria (Table 2; also see Table S1 in the supplemental material). Just one of these groups was identified as AOB-like from the *Nitrosospira* lineage. The other nine groups contained sequences similar to those of different nonnitrifying betaproteobacteria. The NitA/NitB clone library contained sequences from *Nitrosospira* sp. strain Nsp17 and *Nitrosospira* sp. strain Ka3 (31 out of 36 clones) and from four non-AOB groups. The βAMOf/βAMOr library was the most diverse (7 out of 10 groups detected in total), including some sequences (8 out of 38) related to *Nitrosospira* sp. strain Nsp12. The two libraries obtained using the reverse primer Nso1225g were less diverse than the others. Replacing NitB with Nso1225g prevented the amplification of three of the four unspecific groups detected in the NitA/NitB library (*Polynucleobacter*, *Rubrivivax*, and *Thiobacillus*). However, it significantly increased the number of *Methylomonas*-like sequences. Similarly, replacing βAMOr with Nso1225g also decreased the number of unspecific groups but increased the proportion of *Aminomonas*-like sequences.

From the four primer combinations tested with samples from Lake Kinneret, clone libraries prepared with the primer Nso1225g had the lowest proportion of AOB-like sequences. Sequence analysis of the Nso1225 probe has suggested that it is highly specific for βAOB (21, 38, 47). The low specificity of the primer Nso1225g cannot be explained only by the modifi-

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TABLE 2. Recovery of AOB in clone libraries of PCR products prepared with different primer pairs[a]

Source of sample	Primer pair	No. of clones	No. of AOB	% AOB	BLAST hit (*n*)	
					Nitrosospira-like	*Nitrosomonas*-like
Lake Kinneret	βAMOf/βAMOr	38	8	21.1	*Nitrosospira* sp. strain Nsp12 (8)	None
	NitA/NitB	36	31	86.1	*Nitrosospira* sp. strain Nsp17 (30), *Nitrosospira* sp. strain Ka3 (1)	None
	βAMOf/Nso1225g	34	3	8.8	*Nitrosospira* sp. strain Nsp17 (3)	None
	NitA/Nso1225g	38	2	5.3	*Nitrosospira* sp. strain Nsp17 (1), *Nitrosospira* sp. strain Nsp12 (1)	None
Plußsee	βAMOf/βAMOr	93	0	0	None	None
	NitA/NitB	65	41	63.1	*Nitrosospira* sp. strain HB (9) *Nitrosospira* sp. strain Nv6 (1)	*Nitrosomonas* sp. strain Is79A3 (24) *Nitrosomonas* sp. strain Nm86 (5) *Nitrosomonas* sp. strain R7c131 (2)
Baltic Sea, 20 m	βAMOf/βAMOr	81	3	3.7	*Nitrosospira* sp. strain Nsp57 (2) *Nitrosospira* sp. strain III7 (1)	None
	NitA/NitB	91	91	100	*Nitrosospira* sp. strain HB (90) *Nitrosospira* sp. strain Nsp57 (1)	None
Baltic Sea, s/w interface	βAMOf/βAMOr	70	51	72.9	*Nitrosospira* sp. strain Nsp58 (2) *Nitrosospira* sp. strain Nsp12 (1)	*Nitrosomonas* sp. strain NS20 (24) *Nitrosomonas* sp. strain Is79A3 (13) *Nitrosomonas* sp. strain BF16c57 (8) *Nitrosomonas* sp. strain R7c140 (3)
	NitA/NitB	71	71	100	*Nitrosospira* sp. strain Nsp57 (1)	*Nitrosomonas* sp. strain BF16c57 (60) *Nitrosomonas* sp. strain Is79A3 (6) *Nitrosomonas* sp. strain NS20 (1)

[a] The first identified hit in BLAST is given for the *Nitrosospira*- and *Nitrosomonas*-like clones. The number of clones (*n*) is indicated in parentheses. No. of clones, total number of clones screened; no. of AOB, total number of AOB clones obtained; % AOB, percentage of AOB clones; s/w, sediment-water.

cation in the 3′ terminal A, compared to the original Nso1225 sequence, since this modification does not change sequence matching (see Table S6 in the supplemental material). To find a possible explanation for the shift in the amplification spectrum from *Nitrosospira* to *Methylomonas*, sequences from our clones and other sequences from *Methylomonas* were compared with the primers. All the reverse primers showed two to four mismatches with *Methylomonas*-like sequences. In βAMOr, Nso1225, and Nso1225g, the mismatches were concentrated at the 5′ end of the annealing region. In contrast, the two mismatches with NitB were at the 3′ end (at positions 17 and 18) of the annealing region, where they can be more decisive for specificity. This difference might explain the lesser recovery of *Methylomonas*-like sequences in the libraries obtained with NitB than was the case with βAMOr and Nso1225g.

Considering the lack of specificity of the primer Nso1225g observed in the libraries from Lake Kinneret, only the primer combinations NitA/NitB (48) and βAMOf/βAMOr (30) were used with the samples from Lake Plußsee and the Baltic Sea. The comparison of the two clonal libraries prepared from the sediment-water interface of Lake Plußsee produced very contrasting results (Table 2; also see Table S2 in the supplemental material). Though many sequences related to both *Nitrosomonas* and *Nitrosospira* were detected with the primer combination NitA/NitB, not a single AOB-like sequence was detected in the clone library with βAMOf/βAMOr products, which was dominated by sequences related to the betaproteobacterial genus *Variovorax*.

In both samples from the Baltic Sea, AOB-like sequences were detected with both the NitA/NitB and βAMOf/βAMOr primer combinations (Table 2). At the sediment-water interface, *Nitrosomonas*-like sequences corresponded to 100% of the library prepared with the primers NitA/NitB (see Table S3 in the supplemental material). In the clone library with βAMOf/βAMOr products, *Nitrosomonas*-like sequences were also dominant, but another 29% of the sequences were related to deltaproteobacteria. In the sample from a 20-m depth of the Baltic Sea, the library prepared with NitA/NitB products was dominated by *Nitrosospira*-like sequences (see Table S4 in the supplemental material). However, in the library prepared with βAMOf/βAMOr products, only 4% of the sequences were related to AOB. The βAMOf/βAMOr library was dominated by the betaproteobacterial genera *Hydrogenophaga* and *Delftia*.

Analysis of the clone libraries prepared in this study showed that the 16S rRNA gene primers used for the detection of βAOB by PCR differed in their specificity when used with different environmental samples (Table 2). Sequences of βAOB, including those associated with *Nitrosospira* and *Nitrosomonas*, dominated in all clone libraries made from PCR products with the primer combination NitA/NitB (48), while in those prepared with βAMOf/βAMOr (30), non-AOB sequences (*Methylomonas*, *Variovorax*, *Hydrogenophaga*, and *Delftia*) dominated. These results are contradictory to conclusions drawn on the basis of theoretical sequence matching that among all primers used for specific amplification of the 16S rRNA gene from βAOB, βAMOf/βAMOr best fulfilled the criteria of specificity and sensitivity (21, 38). In another independent experimental evaluation for the specific detection of βAOB by PCR and DGGE (29), the primers βAMOf/βAMOr also produced a high proportion of bands from non-AOB compared to results for nested amplification with the primers CTO189f/CTO654r.

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Recovery of different AOB clusters in published studies that used different primer combinations. In order to increase the spectrum of habitats considered in which AOB communities are present, published data from a larger number of habitats were included in the analysis. A total of 24 publications were examined (see Table S5 in the supplemental material), dealing with samples from seawater (3, 11, 12, 16, 33, 35), estuaries (7, 9, 42), freshwater (18), marine or freshwater sediments (10, 30, 41, 42, 44), hypersaline lake (51), soil (6, 25, 26, 34, 41, 43, 44, 53–55), rhizosphere (22, 42), wastewater treatment plants (1, 8, 9, 40), and cultures (48). The comparison of published clone libraries was complemented by an in silico analysis of primer matching using an ARB database updated with all sequences from cultured species in GenBank (see Table S6 in the supplemental material). Despite the importance of sequence analysis for primer design and theoretical evaluation of primer and target interaction, this information alone is not sufficient to predict the outcome of a PCR. Information about the applicability of different primer combinations for the detection of AOB in different environments may be derived from a combined analysis of the retrieval of specific groups of AOB from various environments and sequence matching of the primers used (Table 3).

Amplification of non-AOB sequences is recognized in all publications considered in Table 3, but the proportion of unspecific sequences was given in only a few cases. The primer combinations most frequently used to analyze AOB communities in environmental samples were βAMOf/βAMOr and NitA/NitB and a nested amplification from βAMOf/βAMOr products with the primers CTO189f/CTO654r. Although significant variations in the composition of the AOB communities in the same type of environment may occur, in addition the methods of DNA extraction and amplification (especially the use of nested or direct amplification) can have a strong influence on the composition of the clone libraries. Some tendencies of preferential amplification with different primer combinations can be concluded from the data in Table 3. To simplify the presentation of the results, the cluster designations used by Freitag and Prosser (11) were followed.

The poor amplification of sequences related to *Nitrosospira* cluster 0 in all studies contrasted with the high sequence similarity to all AOB-specific primers. This might indicate a restriction of this group to a few habitats or a very low abundance in nature, which is supported by the observation that sequences related to this group were found in samples from the metalimnion of Lake Kinneret by a nested PCR approach used to improve the detection of this group.

Sequences from *Nitrosospira* clusters 2 and 4 have been amplified with the primers βAMOf/βAMOr and CTO189f/CTO654r from soil and rhizosphere. Strains belonging to these clusters have been isolated from soil, and it has been suggested that they might be specific for this environment (37). Sequences from cluster 2 have not been recovered with the primer combination NitA/NitB, which might reflect the fact that these primers have not been frequently used with soil samples (see Table S5 in the supplemental material). Data from our study showed that sequences related to *Nitrosospira* cluster 4 can also be amplified from freshwater samples with NitA/NitB and βAMOf/βAMOr, contradicting the conclusion that they are apparently restricted to soil habitats (37). Be-

TABLE 3. AOB in published clone libraries of PCR products prepared with different primer combinations[a]

Environment	Sequence detection with primer pair for genus and cluster																																			
	βAMOf/βAMOr													CTO189f/CTO654r												NitA/NitB										
	Nitrosospira					*Nitrosomonas*						*Nitrosospira*					*Nitrosomonas*						*Nitrosospira*					*Nitrosomonas*							
	0	1	2	3	4	U	5	6	7	8	9	Nc	U	0	1	2	3	4	U	5	6	7	8	9	Nc	U	0	1	2	3	4	U	5	6	7	8	9	Nc	U
Seawater		X							X					ND	ND	ND	ND	ND	ND			ND	ND	ND	ND			X				X		X				
Marine sediment		X					X	X	X		X									X							ND	ND	ND	ND	ND	ND	X	ND	ND	ND	ND	ND
Estuary							X	X					X				X		X		X					X						X						
Brackish water	X	X												ND	ND	ND	ND	ND	ND								X	X										
Brackish sediment	X	X					X	X						ND	ND	ND	ND	ND	ND								X	X					X	X				
Freshwater	X				X									ND	ND	ND	ND	ND	ND									X		X	X		X					
Lake sediment								X						ND	ND	ND	ND	ND	ND									X		X			X					
Aquaria	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND									X						ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Salt Lake	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND		X													X				
Wastewater	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND					X		X		X						X		X		X	ND	ND	X				
Soil			X	X	X	X		X	X						X	X	X	X			X	X					ND	ND	ND	ND	ND	ND	ND	X	ND	ND	ND	ND
Rhizosphere				X	X												X	X												X							
Coastal dunes		X	X	X	X			X	X					ND	ND	ND	ND	ND	ND			ND	ND	ND	ND	ND		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Cluster designations according to the work of Freitag and Prosser (11). U, unclustered sequences; Nc, *Nitrosomonas cryotolerans*. "X" indicates the presence of the cluster. ND, environments for which no data were included for a particular primer pair.

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cause they have been detected in the metalimnion besides the water/sediment interface, it is unlikely that they have been washed off from soil habitats.

Nitrosospira cluster 3 was the most common group detected with the primer combinations βAMOf/βAMOr and CTO189f/CTO654r in soil. This cluster also appeared in libraries with the primers NitA/NitB from estuary (7) and wastewater treatment plant (1) sources. We have been able to detect sequences related to *Nitrosospira* cluster 3 in libraries prepared with the primers NitA/NitB and NitA/Nso1225g in samples from freshwater, lake sediment, and rhizosphere. The detection of *Nitrosospira* cluster 3 with the primer NitA contradicts the statement based on theoretical sequence comparison (21) that this primer is not recommended for studying AOB communities due to its low sequence similarity with sequences from *Nitrosospira* cluster 3.

Despite their low sequence similarity with all primers, sequences from *Nitrosomonas oligotropha* (subcluster 6a) and *Nitrosomonas marina* (subcluster 6b) are frequent in clone libraries from a variety of environments, underlining our conclusion that sequence comparison alone is not a reliable indicator for predicting the outcome of a PCR.

Sequences related to *Nitrosomonas* cluster 7 have rarely been detected in natural environments, even when analyzed with the primer combination NitA/NitB, which matches perfectly most of the sequences in cluster 7. It has been suggested that this cluster contains species with preference for eutrophic habitats (20), and more recently the existence of physiological types adapted to extreme environmental conditions has been reported (51). Therefore, habitat adaptation might be an important factor explaining the low frequency of *Nitrosomonas* cluster 7 in the clone libraries from nonextreme environmental samples.

Sequences related to *Nitrosomonas* cluster 8 have rarely been detected in environmental clone libraries, though several cultures belonging to this cluster have been isolated from soil (20). Therefore, the low representation of this cluster in libraries prepared from soil samples with the primers βAMOf/βAMOr and CTO189f/CTO654r is surprising. Sequences related to cluster 8 were amplified from rhizospheric soil by using preamplification with bacterial primers prior to specific PCR with the primers NitA/NitB (Junier et al., unpublished), though NitA has low similarity with all sequences from this cluster.

So far, 16S rRNA gene sequences related to *Nitrosomonas cryotolerans* have not been amplified from environmental samples despite high sequence similarity with all the primers. This fact could reflect a high adaptation of these species to habitats that have not yet been studied in detail.

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REFERENCES

1. **Abd El Haleem, D., F. von Wintzingerode, A. Moter, H. Moawad, and U. B. Göbel.** 2000. Phylogenetic analysis of rhizosphere-associated beta-subclass proteobacterial ammonia oxidizers in a municipal wastewater treatment plant based on rhizoremediation technology. Lett. Appl. Microbiol. **31:** 34–38.
2. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Bano, N., and J. T. Hollibaugh.** 2000. Diversity and distribution of DNA sequences with affinity to ammonia-oxidizing bacteria of the beta subdivision of the class *Proteobacteria* in the Arctic Ocean. Appl. Environ. Microbiol. **66:**1960–1969.
4. **Bothe, H., G. Jost, M. Schloter, B. B. Ward, and K.-P. Witzel.** 2000. Molecular analysis of ammonia oxidation and denitrification in natural environments. FEMS Microbiol. Rev. **24:**673–690.
5. **Brosius, J., M. L. Palmer, P. J. Kennedy, and H. F. Noller.** 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA **75:**4801–4805.
6. **Bruns, M. A., J. R. Stephen, G. A. Kowalchuk, J. I. Prosser, and E. A. Paul.** 1999. Comparative diversity of ammonia oxidizer 16S rRNA gene sequences in native, tilled, and successional soils. Appl. Environ. Microbiol. **65:**2994–3000.
7. **Caffrey, J. M., N. Harrington, I. Solem, and B. B. Ward.** 2003. Biogeochemical processes in a small California estuary. 2. Nitrification activity, community structure and role in nitrogen budgets. Mar. Ecol. Prog. Ser. **248:**27–40.
8. **Calvo, L., X. Vila, C. A. Abella, and L. J. Garcia-Gil.** 2004. Use of the ammonia-oxidizing bacterial-specific phylogenetic probe Nso1225 as a primer for fingerprint analysis of ammonia-oxidizer communities. Appl. Microbiol. Biotechnol. **63:**715–721.
9. **de Bie, M. J. M., A. G. C. L. Speksnijder, G. A. Kowalchuk, T. Schuurman, G. Zwart, J. R. Stephen, O. E. Diekmann, and H. J. Laanbroek.** 2001. Shifts in the dominant populations of ammonia-oxidizing beta-subclass Proteobacteria along the eutrophic Schelde estuary. Aquat. Microb. Ecol. **23:**225–236.
10. **Freitag, T. E., and J. I. Prosser.** 2003. Community structure of ammonia-oxidizing bacteria within anoxic marine sediments. Appl. Environ. Microbiol. **69:**1359–1371.
11. **Freitag, T. E., and J. I. Prosser.** 2004. Differences between betaproteobacterial ammonia-oxidizing communities in marine sediments and those in overlying water. Appl. Environ. Microbiol. **70:**3789–3793.
12. **Grommen, R., L. Dauw, and W. Verstraete.** 2005. Elevated salinity selects for a less diverse ammonia-oxidizing population in aquarium biofilms. FEMS Microbiol. Ecol. **52:**1–11.
13. **Hastings, R. C., M. T. Ceccherini, N. Miclaus, J. R. Saunders, M. Bazzicalupo, and A. J. McCarthy.** 1997. Direct molecular biological analysis of ammonia oxidizing bacteria populations in cultivated soil plots treated with swine manure. FEMS Microbiol. Ecol. **23:**45–54.
14. **Head, I. M., W. D. Hiorns, T. M. Embley, A. J. McCarthy, and J. R. Saunders.** 1993. The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J. Gen. Microbiol. **139:**1147–1153.
15. **Hiorns, W. D., R. C. Hastings, I. M. Head, A. J. McCarthy, J. R. Saunders, R. W. Pickup, and G. H. Hall.** 1995. Amplification of 16S ribosomal RNA genes of autotrophic ammonia-oxidizing bacteria demonstrates the ubiquity of nitrosospiras in the environment. Microbiology **141:**2793–2800.
16. **Hollibaugh, J. T., N. Bano, and H. W. Ducklow.** 2002. Widespread distribution in polar oceans of a 16S rRNA gene sequence with affinity to *Nitrosospira*-like ammonia-oxidizing bacteria. Appl. Environ. Microbiol. **68:** 1478–1484.
17. **Juretschko, S., G. Timmermann, M. Schmid, K. H. Schleifer, A. Pommerening-Röser, H. P. Koops, and M. Wagner.** 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. **64:**3042–3051.
18. **Kim, O. S., P. Junier, J. F. Imhoff, and K. P. Witzel.** 2006. Comparative analysis of ammonia-oxidizing bacterial communities in two lakes in North Germany and the Baltic Sea. Arch. Hydrobiol. **167:**335–350.
19. **Konneke, M., A. E. Bernhard, J. R. de la Torre, C. B. Walker, J. B. Waterbury, and D. A. Stahl.** 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature **437:**543–546.
20. **Koops, H.-P., and A. Pommerening-Röser.** 2001. Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. FEMS Microbiol. Ecol. **37:**1–9.
21. **Koops, H.-P., U. Purkhold, A. Pommerening-Röser, G. Timmermann, and M. Wagner.** 2003. The lithoautotrophic ammonia-oxidizing bacteria. *In* M. Dworkin (ed.), The prokaryotes. Springer-Verlag, New York, NY.
22. **Kowalchuk, G. A., P. L. E. Bodelier, G. Hans, J. Heilig, J. R. Stephen, and H. J. Laanbroek.** 1998. Community analysis of ammonia-oxidising bacteria, in relation to oxygen availability in soil and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridization. FEMS Microbiol. Ecol. **27:**339–350.
23. **Kowalchuk, G. A., and J. R. Stephen.** 2001. Ammonia-oxidizing bacteria: a model for molecular microbial ecology. Annu. Rev. Microbiol. **55:**485–529.
24. **Kowalchuk, G. A., J. R. Stephen, W. De Boer, J. I. Prosser, T. M. Embley, and J. W. Woldendorp.** 1997. Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class *Proteobacteria* in coastal sand dunes by dena-

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turing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl. Environ. Microbiol. **63:**1489–1497.
25. **Kowalchuk, G. A., A. W. Stienstra, G. H. Heilig, J. R. Stephen, and J. W. Woldendorp.** 2000. Molecular analysis of ammonia-oxidising bacteria in soil of successional grasslands of the Drentsche A (The Netherlands). FEMS Microbiol. Ecol. **31:**207–215.
26. **Laverman, A. M., A. G. Speksnijder, M. Braster, G. A. Kowalchuk, H. A. Verhoef, and H. W. Van Verseveld.** 2001. Spatiotemporal stability of an ammonia-oxidizing community in a nitrogen-saturated forest soil. Microb. Ecol. **42:**35–45.
27. **Leininger, S., T. Urich, M. Schloter, L. Schwark, J. Qi, G. W. Nicol, J. I. Prosser, S. C. Schuster, and C. Schleper.** 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature **442:**806–809.
28. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüßmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K. H. Schleifer.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32:**1363–1371.
29. **Mahmood, S., T. E. Freitag, and J. I. Prosser.** 2006. Comparison of PCR primer-based strategies for characterization of ammonia oxidizer communities in environmental samples. FEMS Microbiol. Ecol. **56:**482–493.
30. **McCaig, A. E., T. M. Embley, and J. I. Prosser.** 1994. Molecular analysis of enrichment cultures of marine ammonia oxidisers. FEMS Microbiol. Lett. **120:**363–367.
31. **Mobarry, B. K., M. Wagner, V. Urbain, B. E. Rittmann, and D. A. Stahl.** 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. **62:**2156–2162.
32. **Nocker, A., M. Burr, and A. K. Camper.** 2007. Genotypic microbial community profiling: a critical technical review. Microb. Ecol. **54:**276–289.
33. **O'Mullan, G. D., and B. B. Ward.** 2005. Relationship of temporal and spatial variabilities of ammonia-oxidizing bacteria to nitrification rates in Monterey Bay, California. Appl. Environ. Microbiol. **71:**697–705.
34. **Phillips, C. J., D. Harris, S. L. Dollhopf, K. L. Gross, J. I. Prosser, and E. A. Paul.** 2000. Effects of agronomic treatments on structure and function of ammonia-oxidizing communities. Appl. Environ. Microbiol. **66:**5410–5418.
35. **Phillips, C. J., Z. Smith, T. M. Embley, and J. I. Prosser.** 1999. Phylogenetic differences between particle-associated and planktonic ammonia-oxidizing bacteria of the beta subdivision of the class *Proteobacteria* in the Northwestern Mediterranean Sea. Appl. Environ. Microbiol. **65:**779–786.
36. **Pommerening-Röser, A., G. Rath, and H.-P. Koops.** 1996. Phylogenetic diversity within the genus *Nitrosomonas*. Syst. Appl. Microbiol. **19:**344–351.
37. **Prosser, J. I., and T. M. Embley.** 2002. Cultivation-based and molecular approaches to characterization of terrestrial and aquatic nitrifiers. Antonie van Leeuwenhoek **81:**165–179.
38. **Purkhold, U., A. Pommerening-Röser, S. Juretschko, M. C. Schmid, H. P. Koops, and M. Wagner.** 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.
39. **Purkhold, U., M. Wagner, G. Timmermann, A. Pommerening-Röser, and H. P. Koops.** 2003. 16S rRNA and *amoA*-based phylogeny of 12 novel betaproteobacterial ammonia-oxidizing isolates: extension of the dataset and proposal of a new lineage within the nitrosomonads. Int. J. Syst. Evol. Microbiol. **53:**1485–1494.
40. **Rowan, A. K., J. R. Snape, D. Fearnside, M. R. Barer, T. P. Curtis, and I. M. Head.** 2003. Composition and diversity of ammonia-oxidising bacterial communities in wastewater treatment reactors of different design treating identical wastewater. FEMS Microbiol. Ecol. **43:**195–206.
41. **Smith, Z., A. E. McCaig, J. R. Stephen, T. M. Embley, and J. I. Prosser.** 2001. Species diversity of uncultured and cultured populations of soil and marine ammonia oxidizing bacteria. Microb. Ecol. **42:**228–237.
42. **Speksnijder, A. G., G. A. Kowalchuk, K. Roest, and H. J. Laanbroek.** 1998. Recovery of a *Nitrosomonas*-like 16S rDNA sequence group from freshwater habitats. Syst. Appl. Microbiol. **21:**321–330.
43. **Stephen, J. R., G. A. Kowalchuk, M. A. V. Bruns, A. E. McCaig, C. J. Phillips, T. M. Embley, and J. I. Prosser.** 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.
44. **Stephen, J. R., A. E. McCaig, Z. Smith, J. I. Prosser, and T. M. Embley.** 1996. Molecular diversity of soil and marine 16S rRNA gene sequences related to beta-subgroup ammonia-oxidizing bacteria. Appl. Environ. Microbiol. **62:**4147–4154.
45. **Teske, A., E. Alm, J. M. Regan, S. Toze, B. E. Rittmann, and D. A. Stahl.** 1994. Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. J. Bacteriol. **176:**6623–6630.
46. **Treusch, A. H., S. Leininger, A. Kletzin, S. C. Schuster, H. P. Klenk, and C. Schleper.** 2005. Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. Environ. Microbiol. **7:**1985–1995.
47. **Utaker, J. B., and I. F. Nes.** 1998. A qualitative evaluation of the published oligonucleotides specific for the 16S rRNA gene sequences of the ammonia-oxidizing bacteria. Syst. Appl. Microbiol. **21:**72–88.
48. **Voytek, M. A., and B. B. Ward.** 1995. Detection of ammonium-oxidizing bacteria of the beta-subclass of the class *Proteobacteria* in aquatic samples with the PCR. Appl. Environ. Microbiol. **61:**1444–1450.
49. **Wagner, M., R. Amann, H. Lemmer, and K. H. Schleifer.** 1993. Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. Appl. Environ. Microbiol. **59:**1520–1525.
50. **Wagner, M., G. Rath, R. Amman, H.-P. Koops, and K. H. Schleifer.** 1995. *In situ* identification of ammonia-oxidizing bacteria. Syst. Appl. Microbiol. **18:**251–264.
51. **Ward, B. B., D. P. Martino, M. C. Diaz, and S. B. Joye.** 2000. Analysis of ammonia-oxidizing bacteria from hypersaline Mono Lake, California, on the basis of 16S rRNA sequences. Appl. Environ. Microbiol. **66:**2873–2881.
52. **Ward, B. B., M. A. Voytek, and K. P. Witzel.** 1997. Phylogenetic diversity of natural populations of ammonia oxidizers investigated by specific PCR amplification. Microb. Ecol. **33:**87–96.
53. **Webster, G., T. M. Embley, T. E. Freitag, Z. Smith, and J. I. Prosser.** 2005. Links between ammonia oxidizer species composition, functional diversity and nitrification kinetics in grassland soils. Environ. Microbiol. **7:**676–684.
54. **Webster, G., T. M. Embley, and J. I. Prosser.** 2002. Grassland management regimens reduce small-scale heterogeneity and species diversity of beta-proteobacterial ammonia oxidizer populations. Appl. Environ. Microbiol. **68:**20–30.
55. **Wheatley, R. E., S. Caul, D. Crabb, T. J. Daniell, B. S. Graffiths, and K. Ritz.** 2003. Microbial population dynamics related to temporal variations in nitrification in three arable fields. Eur. J. Soil Sci. **54:**707–714.

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