RESEARCH ARTICLE

Comparative *in silico* analysis of PCR primers suited for diagnostics and cloning of ammonia monooxygenase genes from ammonia-oxidizing bacteria

Pilar Junier[1,2], Ok-Sun Kim[2], Verónica Molina[2], Petra Limburg[2], Thomas Junier[3], Johannes F. Imhoff[4] & Karl-Paul Witzel[2]

[1]Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland; [2]Max-Planck Institute for Limnology, Ploen, Germany; [3]University of Geneva, Geneva, Switzerland; and [4]Leibniz-Institute of Marine Sciences at the University of Kiel, Kiel, Germany

Correspondence: Pilar Junier, EPFL ENAC ISTE EML, CE 1 644 (Centre Est), Station 6, CH-1015 Lausanne, Switzerland. Tel.: +41 21 693 63 96; fax: +41 21 693 62 05; e-mail: pilar.junier@epfl.ch

Received 5 July 2007; revised 21 November 2007; accepted 23 November 2007.
First published online 1 February 2008.

DOI:10.1111/j.1574-6941.2007.00437.x

Editor: Michael Wagner

Keywords
Ammonia-oxidizing bacteria; PCR primers; *amoC*; *amoA*; *amoB*.

Abstract

Over recent years, several PCR primers have been described to amplify genes encoding the structural subunits of ammonia monooxygenase (AMO) from ammonia-oxidizing bacteria (AOB). Most of them target *amoA*, while *amoB* and *amoC* have been neglected so far. This study compared the nucleotide sequence of 33 primers that have been used to amplify different regions of the *amoCAB* operon with alignments of all available sequences in public databases. The advantages and disadvantages of these primers are discussed based on the original description and the spectrum of matching sequences obtained. Additionally, new primers to amplify the almost complete *amoCAB* operon of AOB belonging to *Betaproteobacteria* (betaproteobacterial AOB), a primer pair for DGGE analysis of *amoA* and specific primers for gammaproteobacterial AOB, are also described. The specificity of these new primers was also evaluated using the databases of the sequences created during this study.

Introduction

Ammonia-oxidizing bacteria (AOB) are chemolithoautotrophic Gram-negative proteobacteria that fix CO_2 with the reducing power obtained from ammonia oxidation (Prosser, 1989). They belong to two monophyletic lineages: *Nitrosomonas* spp. (including *Nitrosococcus mobilis*) and *Nitrosospira* spp. (including *Nitrosolobus* and *Nitrosovibrio*) form a closely related clade within the beta phylum (betaproteobacterial AOB) of proteobacteria, whereas *Nitrosococcus oceani* is affiliated to the gamma phylum (gammaproteobacterial AOB) of proteobacteria (Head *et al.*, 1993; Purkhold *et al.*, 2000; Purkhold *et al.*, 2003).

Characterization of the species composition and diversity of AOB communities in nature has been hampered for a long time by difficulties in the isolation and culture of these microorganisms. Analysis of AOB communities has become accomplishable by applying culture-independent molecular approaches, which are based on the amplification of 16S rRNA genes by PCR (Bothe *et al.*, 2000; Kowalchuk & Stephen, 2001) or the detection of 16S rRNA by FISH (Wagner *et al.*, 1993, 1995; Mobarry *et al.*, 1996). 16S rRNA genes are good phylogenetic markers, but are not necessarily related to the physiology of the target organisms (Kowalchuk & Stephen, 2001; Calvo & Garcia-Gil, 2004). Therefore, functional markers such as the genes encoding for key enzymes involved in ammonia-oxidation provide an alternative in ecological studies (Rotthauwe *et al.*, 1997). Diversity studies of AOB based on the sequence analysis of one of these genes, *amoA*, have shown a high resolution in separating closely related strains (Rotthauwe *et al.*, 1997; Alzerreca *et al.*, 1999; Aakra *et al.*, 2001; Norton *et al.*, 2002).

Ammonia monooxygenase (AMO) is a membrane-bound multiple subunit enzyme responsible for the conversion of ammonia to hydroxylamine (Hyman & Arp, 1992). The structural subunits of AMO in AOB are encoded by the genes *amoC*, *amoA* and *amoB*, which are organized in one operon (Norton *et al.*, 2002). The physical organization of the operon seems to be conserved in all AOB; multiple copies have been reported for betaproteobacterial AOB (Norton *et al.*, 2002), whereas so far it seems that in

gammaproteobacterial AOB occurs as a single copy (Alzerreca *et al.*, 1999).

Since the publication of the first *amoA* sequence of *Nitrosomonas europaea* (McTavish *et al.*, 1993), the number of partial and full-length sequences available in public databases has increased significantly. Several PCR primers to amplify *amoA* have been published (Holmes *et al.*, 1995; Sinigalliano *et al.*, 1995; Rotthauwe *et al.*, 1997; Juretschko *et al.*, 1998; Nold *et al.*, 2000; Purkhold *et al.*, 2000; Hoshino *et al.*, 2001; Nicolaisen & Ramsing, 2002; Norton *et al.*, 2002; Okano *et al.*, 2004). The analysis of AMO-encoding genes has been extended to *amoC* and *amoB* (Purkhold *et al.*, 2000; Norton *et al.*, 2002; Calvo & Garcia-Gil, 2004), and more recently functional genes homologous to those in AOB have been described in Archaea (Konneke *et al.*, 2005; Treusch *et al.*, 2005). Some of these primers were designed when only a few sequences were available. Considering the new sequence information accumulated in recent years, including the complete genomes of *Nitrosomonas europaea* (Chain *et al.*, 2003), *Nitrosococcus oceani* (Klotz *et al.*, 2006) and *Nitrosospira multiformis,* sequence analysis can contribute to estimate the advantages and failures of the available primers, and to assist the development of new strategies to study the structure of AOB communities. In this study all available *amoCAB* sequences from recognized AOB species, and whenever possible the sequences from uncultured clones, were used to characterize the published PCR primers and to propose new primers for the amplification of the *amoCAB* operon.

Materials and methods

Sequences and alignments

For *in silico* analyses, the nucleotide sequences of *amo* genes were downloaded from GenBank using ENTREZ (http://www.ncbi.nlm.nih.gov/). Protein sequences were retrieved from Swissprot using EXPASY (http://www.expasy.org). The analyzed sequences were: (a) 16 sequences of *amoC* from both beta- and gammaproteobacterial AOB, (b) eight sequences of the related subunit of the particulate methane monooxygenase (*pmoC*), (c) one *amoC* sequence from the recently described ammonia-oxidizing archaeon *Candidatus Nitrosopumilus maritimus* (Konneke *et al.*, 2005), (d) 32 *amoB* sequences from beta- and gammaproteobacterial AOB; (e) seven *pmoB* sequences from methane-oxidizing bacteria (MOB), (f) two *amoB* sequences from crenarchaeota and (g) 2669 sequences of *amoA* and the related α subunit of the particulate methane monooxygenase (*pmoA*) from cultured and uncultured AOB. Although *amoA* sequences from crenarchaeota were considered, they differed widely and were excluded from the analysis.

The *amoC*, *pmoC*, *amoB*, *pmoB*, *amoA* and *pmoA* sequences were integrated in ARB (Ludwig *et al.*, 2004). A database of complete and partial sequences of *amoA* from recognized AOB species was also prepared in ARB. Before the analysis, the sequences were verified manually and those including STOP codons or erroneous starting points were omitted. To simplify the presentation of the results, sequence similarity is shown only for *amoA* sequences from cultured AOB (11 different phylogenetic clusters) and 10 *pmoA* sequences. The complete databases are available at http://cegg.unige.ch/ammoniaoxigenase. Sequences were aligned using CLUSTALW included in ARB.

Primers

To simplify the comparison between primers that had been designed in different studies, this study proposes a standardized designation system according to the name of the targeted gene, followed by information on the position and orientation of the primers. An example of such designation is as follows: amoA31f in which '*amoA*' indicates the gene targeted, '*31*' the position in the alignment and '*f*' the direction of the primers (forward). Additional letters at the end of the designation indicate modifications such as shorter versions (s), wobble positions (IUPAC code), probe for FISH (p) or primer specific for gammaproteobacterial AOB (Gam). The new designation is always given in parenthesis after the original designation of the primer [e.g. AMO-F (amoA21f)].

Analysis of the primers was carried out using the software OLIGO 6.0 (Table 1). The position of each primer was determined after alignment of all the sequences in ARB. Specificity was evaluated using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) for short, nearly exact matches and also MATCH PROBE in ARB. Because the different T_m values in the presence of several mismatches calculated by OLIGO (Table 1) do not take into account the position of the mismatch, additional analyses were carried out with MATCH PROBE in ARB. The MATCH PROBE subroutine of ARB calculates two different parameters for specificity: number of mismatches and weight of the mismatches. The last parameter depends on the number, position and kind of mismatches. A maximum number of five mismatches was allowed in the analysis. New primers were designed by visual inspection of the multiple alignments or using the software GENEFISHER (http://bibiserv.techfak.uni-bielefeld.de/genefisher/). The newly designed primers were also analyzed with OLIGO v.6.0 (Table 1).

Results and discussion

Sequence analysis of *amoA* primers

Sequence matching of the *amoA* primers was analyzed in the ARB database prepared in this study. The complete

Table 1. Primers analyzed in this study

Gene	New designation	Original designation	Sequence 5′–3′	Position	Length (bp)	Deg.	T_m (°C) oligo with different number of mm 0	1	2	3	4	Loop T_m (°C)	References
amoA	amoA21f	AMO-F	AGA AAT CCT GAA AGC GGC	21–38	18	1	62.2	55.5	48.9	42.2	35.5	N	Sinigalliano *et al*. (1995)
	amoA34f		GCG GCR AAA ATG CCG CCG GAA GCG	34–57	24	1	86.4	81.4	76.4	71.4	66.4	105	Molina *et al*. (2007)
	amoA40f	AMO-F2	AAG ATG CCG CCG GAA GC	40–56	17	1	68.7	61.6	54.6	47.5	40.4	N	Juretschko *et al*. (1998)
	amoA49f		GAG GAA GCT GCT AAA GTC	49–66	18	1	53.6	46.9	40.2	33.6	26.9	N	This study
	amoA60r	304R	TAY CGC TTC CGG CGG CAT TTT CGC CGC	34–60	27	2	75.8	70.1	64.4	58.7	53.0	67.0	Norton *et al*. (2002)
	amoA121f	amoA-3F	ACC TAC CAC ATG CAC TT	121–137	17	1	51.0	44.0	36.9	29.9	22.8	N	Webster *et al*. (2002)
	amoA151f	A189	GGN GAC TGG GAC TTC TGG	151–168	18	4	59.0	52.4	45.7	39.0	32.4	N	Holmes *et al*. (1995)
	amoA154f	301F	GAC TGG GAC TTC TGG CTG GAC TGG AA	154–179	26	1	67.9	62.2	56.5	50.8	45.1	N	Norton *et al*. (2002)
	amoA154fs		GAC TGG GAC TTC TGG	154–168	15	1	46.3	38.3	30.3	22.3	14.3	N	This study
	amoA187f	amoA-1FF	CAA TGG TGG CCG GTT GT	187–203	17	1	64.4	57.3	50.2	43.2	36.1	16.0	Hoshino *et al*. (2001)
	amoA310f	amoA-3F	GGT GAG TGG GYT AAC MG	310–326	17	4	51.1	44.0	36.9	29.9	22.8	N	Purkhold *et al*. (2000)
	amoA332f	amoA-1F	GGG GTT TCT ACT GGT GGT	332–349	18	1	58.3	51.6	45.0	38.3	31.6	N	Rotthauwe *et al*. (1997)
	amoA332fHY	amoA1F mod	GGG GHT TYT ACT GGT GGT	332–349	18	6	58.8	52.1	45.4	38.8	32.1	N	Stephen *et al*. (1999)
	amoA337p	A337	TTC TAC TGG TGG TCR CAC TAC CCC ATC AAC T	337–367	31	2	56.0	50.2	44.5	38.8	33.1	N	Okano *et al*. (2004)
	amoA359rC	amoA-4R	GGG TAG TGC GAC CAC CAG TA	340–359	20	1	65.2	59.2	53.2	47.2	41.2	30.0	Webster *et al*. (2002)
	amoA627r		CGT ACC TTT TTC AAC CAT CC	608–627	20	1	62.0	56.0	50.0	44.0	38.0	N	This study
	amoA665r	AMO-R2	GCT GCA ATA ACT GTG GTA	648–665	18	1	53.4	46.7	40.1	33.4	26.7	N	Juretschko *et al*. (1998)
	amoA680r	A682 mod	AAV GCV GAG AAG AAW GC	664–680	17	18	51.5	44.4	37.4	30.3	23.3	N	Nold *et al*. (2000)
	amoA681r	A682	GAA SGC NGA GAA GAA SGC	664–681	18	16	54.4	47.8	41.1	34.5	27.8	N	Holmes *et al*. (1995)
	amoA686r	AMO-R	GAT ACG AAC GCA GAG AAG	669–686	18	1	54.9	48.3	41.6	34.9	28.3	N	Sinigalliano *et al*. (1995)
	amoA820r	AmoA-2R′	CCT CKG SAA AGC CTT CTT C	802–820	19	4	56.1	49.8	43.5	37.2	30.9	3.0	Okano *et al*. (2004)
	amoA822r	amoA-2R	CCC CTC KGS AAA GCC TTC TTC	802–822	21	4	65.0	59.2	53.5	47.8	42.1	3.0	Rotthauwe *et al*. (1997)
	amoA822rTC	amoA-2R-TC	CCC CTC TGC AAA GCC TTC TTC	802–822	21	1	70.2	64.5	58.7	47.3	41.6	3.0	Nicolaisen & Ramsing (2002)
	amoA822rTG	amoA-2R-TG	CCC CTC TGG AAA GCC TTC TTC	802–822	21	1	69.2	63.5	57.8	52.1	46.3	11.0	Okano *et al*. (2004)
	amoA828r	302R	TTT GAT CCC CTC TGG AAA GCC TTC TTC	802–828	27	1	70.2	64.4	58.7	53.0	47.3	30.0	Norton *et al*. (2002)
amoB	amoB44r	amoB-4R	GCT AGC CAC TTT CTG G	29–44	16	1	51.9	44.4	36.9	29.4	21.9	41.0	Purkhold *et al*. (2000)
	amoB160f	amoBMf	TGG TAY GAC ATK AWA TGG	160–177	18	8	47.0	40.3	33.6	27.0	20.3	N	Calvo & Garcia-Gil (2004)
	amoB506r	308R	TCC CAG CTK CCG GTR ATG TTC ATC C	482–506	25	4	68.8	63.1	57.4	51.6	45.9	N	Norton *et al*. (2002)
	amoB660r	amoBMr	RCG SGG CAR GAA CAT SGG	643–660	18	16	62.8	56.1	49.5	42.8	36.1	N	Calvo & Garcia-Gil (2004)
	amoB1179r		CCA AAR CGR CTT TCC GG	1164–1179	17	4	61.0	53.9	46.9	39.8	32.7	N	This study
	amoB1179rGam		GCA AAG CGG CTG TCT GG	1164–1179	17	1	64.8	57.8	50.7	43.7	36.6	N	This study
amoC	amoC58f		CTA YGA CAT GTC RCT GTG G	58–72	19	4	51.5	45.1	38.8	32.5	26.2	N	This study
	amoC763f	305F	GTG GTT TGG AAC RGI CAR AGC AAA	763–786	21	16	61.8	56.1	50.4	44.7	39.0	N	Norton *et al*. (2002)

New primer designations consider: target gene (amo followed by A, B or C), position in the alignment and orientation (forward, f; reverse, r. Modifications of the original primer sequence are shown in IUPAC code after the letter indicating the orientation of the primer. Other designations: p = probe for FISH; s = shorter version; Gam = specific for gamma-AOB. For *amoA* the positions were defined according to the sequence of *Nitrosomonas europaea* (L08050). For *amoB* and *amoC* the positions were defined according to the sequences of the *Nitrosomonas europaea* genome (BX321859). Melting temperature was calculated by nearest neighbor method. Deg. = number of different sequences due to wobble positions. mm = number of mismatched positions. N = no loops detected.

alignment extended over 829 nucleotide positions, which were numbered according to the sequence of *Nitrosomonas europaea* (L08050). The majority of the *amoA* sequences were found in the region between positions 340 and 802. Therefore, the comparison of primers annealing outside of this region was limited to only a few sequences from the following clusters: *Nitrosospira* cluster 3, *Nitrosomonas europaea*, *Nitrosomonas oligotropha*, *Nitrosomonas cryotolerans*, gammaproteobacterial AOB and *Methylococcus capsulatus* (Table 2).

The primer pair AMO-F (amoA21f) and AMO-R (amoA686r) (Sinigalliano *et al.*, 1995), which had been derived from one sequence of *Nitrosomonas europaea* available at that time, proved to be highly specific for the *Nitrosomonas europaea* cluster (Table 2). In the GenBank search, the forward primer AMO-F (amoA21f) matched perfectly sequences from *Nitrosomonas europaea*. In contrast, AMO-F (amoA21f) has three to five mismatches with some sequences of *Nitrosospira* cluster 3, and more than five mismatches with *Nitrosospira multiformis*, two sequences from the *Nitrosomonas oligotropha* cluster, *Nitrosomonas cryotolerans*, MOB and gammaproteobacterial AOB. Additionally, the comparison with clonal sequences from uncultured organisms showed that this primer has five mismatches to another region of *pmoA*. The reverse primer AMO-R (amoA686r) matched perfectly with only three sequences of the *Nitrosomonas europaea* cluster, but possessed two to more than five mismatches with other sequences of this cluster. AMO-R (amoA686r) also has two to four mismatches with almost all sequences from cultured betaproteobacterial AOB, 1190 sequences from uncultured betaproteobacterial AOB and *pmoA* from *Methylococcus capsulatus*. This primer has more than five mismatches with all other MOB and *amoA* of gammaproteobacterial AOB. According to this study's sequence analysis (Table 2), the AMO-F (amoA21f) and AMO-R (amoA686r) pair may be suitable to amplify AOB closely related to *Nitrosomonas europaea* and to exclude other AOB groups under stringent PCR conditions. An experimental evaluation (Sinigalliano *et al.*, 1995) had shown that this primer pair can also amplify *amoA* from *Nitrosomonas cryotolerans* and *Nitrosococcus oceani*, but this conclusion is not supported by the *in silico* evaluation and can only be explained by the use of PCR conditions favoring low specificity.

The primer pair AMO-F2 (amoA40f) and AMO-R2 (amoA665r) (Juretschko *et al.*, 1998) was published to increase the sensitivity of *amoA* detection using a nested PCR approach from templates prepared with the primers AMO-F (amoA21f) and AMO-R (amoA686r) (Sinigalliano *et al.*, 1995), considered above. AMO-F2 (amoA40f) matches perfectly eight of the 14 sequences analyzed (both *Nitrosospira* and *Nitrosomonas* spp.) and have one mismatch with *Nitrosospira* sp. NpAV, two with *Nitrosospira multiformis*, three with *Nitrosomonas cryotolerans*, and more than five with MOB or gammaproteobacterial AOB. The sequence analysis suggests that AMO-F2 (amoA40f) may be suitable to target betaproteobacterial AOB in general. In contrast, the primer AMO-R2 (amoA665r) seems to match sequences from the *Nitrosomonas europaea* cluster (including environmental clones), better than other clusters, matching perfectly only three sequences from the *Nitrosomonas europaea* cluster (Table 2). AMO-R2 (amoA665r) has four high-weighted mismatches to sequences from the *Nitrosospira* lineage and one to four mismatches with different weight with other *Nitrosomonas* sequences. Because of the restricted spectrum of matches of AMO-R2 (amoA665r), the authors conclude that AMO-F2 (amoA40f) may be suitable as a general primer for amplifying betaproteobacterial AOB, but it should be combined with another reverse primer to accomplish this goal. Although the AMO-F2 (amoA40f) and AMO-R2 (amoA775r) pair was originally designed for nested amplification from products prepared with the primers AMO-F (amoA21f) and AMO-R (amoA686r) in order to increase PCR sensitivity, this approach seems to have limited applicability considering that the primer pair used in the first round of PCR (AMO-F and AMO-R) appear to be biased for amplification of *Nitrosomonas europaea*.

Recently, several regions for primer design have been identified based on reverse translation of protein alignment in the *amoCAB* operon (Norton *et al.*, 2002). The primer 304R (amoA60r) is located near the 5′ end of *amoA* and allows, in combination with the primer 305F (amoC763f), the amplification of the intergenic region between *amoC* and *amoA*. This primer does not perfectly match any of the *amoA* sequences of cultured AOB (Table 2), having three to four mismatches of high weight in all cases examined. Additionally, the primer 304R (amoA60r) possesses a very stable loop structure (Table 1), which is not desirable for PCR. The experimental evaluation (Norton *et al.*, 2002) showed that 304R (amoA60r), in combination with 305F (amoC763f), amplified the variable intergenic region of *Nitrosospira* sp. NpAv, *Nitrosospira briensis*, *Nitrosospira* sp. 39–19, *Nitrosospira tenuis*, *Nitrosospira multiformis*, *Nitrosomonas europaea*, *Nitrosomonas eutropha*, *Nitrosomonas* sp. AL212, *Nitrosomonas* sp. JL21, *Nitrosomonas* sp. GH22 and *Nitrosomonas cryotolerans*. However, according to the sequence analysis, this should be only possible under low specificity of PCR (Table 1). This intergenic region can be relevant for community studies because the size of the products obtained from each species is different and the nucleotide sequence is highly variable. However, modification of the primers (for example shortening of primers or designing new primers) might be considered for application with environmental samples.

Table 2. Comparison of the primer sequences with the ARB database

	Strain	Accession	Original designation: AMO-F		AMO-F2		304R		A189	301F		amoA-1FF	amoA-3F	amoA-1F	amoA-1F	A337		AMO-R2	A682 mod	A682	AMO-R	amoA-2R′	amoA-2R	amoA-2RTG	amoA-2RTC	302R
			New designation: amoA21f	**amoA34f**	amoA40f	**amoA49f**	amoA60r	**amoA121f**	amoA151f	amoA154f	**amoA154fs**	amoA187f	amoA310f	amoA332f	amoA332fHY	amoA337p	**amoA359rC**	**amoA627r**	amoA665r	amoA680r	amoA681r	amoA686r	amoA820r	amoA822r	amoA822rTG	amoA822rTC	amoA828r
Cluster 0	*Nitrosospira* sp. III2	AJ298694																4	3	0	2					
	Nitrosospira sp. Nsp5	AY123834																4	3	0	2					
	Nitrosospira sp. Nsp12	AY123823																4	3	0	2					
	Nitrosospira sp. 40Kl	AJ298687																4	3	0	2					
Cluster 2	*Nitrosospira* sp. B6	AJ298690																4	3	0	2					
	Nitrosospira sp. III7	AJ298695																4	3	0	2					
	Nitrosospira sp. O4	AJ298723																4	3	0	2					
	Nitrosospira sp. O13	AJ298722																4	3	0	2					
	Nitrosospira sp. L115	AJ298698																4	3	0	3					
Cluster 3	*Nitrosospira briensis*	U76553	3	3	0		4	0	0	0	0	1		1	0	1	0			4		3	2	2	3	2
	Nitrosospira multiformis	AF042171		3	2		3	0	0	1	0	2	4	2	2	2	0	4	2	1	2	3	2	2	3	2
	Nitrosospira multiformis	AY177933						0	0	1	0	2	4	0	1	1	0		2	2	2					
	Nitrosospira tenuis	AY123824																4	3	2	3					
	Nitrosovibrio tenuis	U76552	5	2	0		3	0	0	0	0	1		1	2	4	0	4	4	3		1	0	0	1	0
	Nitrosospira sp.	U31655										1		2	2	2	0									
	Nitrosospira sp. 24C	AJ298685																4	2	2	3					
	Nitrosospira sp. A16	AJ298688																4	2	2	3					
	Nitrosospira sp. AF	AJ298689																4	2	2	3					
	Nitrosospira sp. L115	AY123817																4	2	2	3					
	Nitrosospira sp. L13	AJ238542																	4	1	2					
	Nitrosospira sp. LT1FMf	AY189144														2	0			4		2	1	1	2	
	Nitrosospira sp. LT2MFa	AY189145														2	0			4		2	1	1	2	
	Nitrosospira sp. NI5	AY123832																4	4	3	3					
	Nitrosospira sp. NpAV	AF032438	5	3	1		4	0	0	1	0	0		0	1	2	0	4	3	1	3	3	2	2	3	2
	Nitrosospira sp. Nsp1	AY123828																		4						
	Nitrosospira sp. Nsp2	AJ298719																3	2	1	2					
	Nitrosospira sp. Nsp2	AY123822																	2	1	2					
	Nitrosospira sp. Nsp17	AY123825																4	2	2	3					
	Nitrosospira sp. Nsp40	AY123840																		4						
	Nitrosospira sp. Nsp62	AY123837																		4						
	Nitrosospira sp. Nsp65	AY123839																	3	2	2					
	Nitrosospira sp. Nsp65	AY123838																3	3	2	2					
	Nitrosospira sp. Np 39-19	AF042170						0	0	0	0	1	4	0	0	2	0	4	4	4		1	0	0	1	1
	Nitrosospira sp. Nv6	AY123826																4	4	3						
Cluster 4	*Nitrosospira* sp. CT2F	AY189143														1	0		3	1	3	2	1	1	2	
	Nitrosospira sp. Ka3	AJ298696																	3	1	3					
	Nitrosospira sp. Ka4	AJ298697																	3	1	3					
No cluster	*Nitrosospira* sp. Nsp41	AY123833																4	3	1	3					
	Nitrosospira sp. Nsp58	AY123836																	2	1	3					
	Nitrosospira sp. Nsp57	AY123835																4	4	4						
	Nitrosospira sp. NI20	AJ298703																4	4	3	4					

Table 2. Continued.

	Strain	Accession	Original designation																												
				AMO-F		AMO-F2		304R		A189	301F		amoA-1FF	amoA-3F	amoA-1F	amoA-1F	A337		AMO-R2	A682 mod	A682	AMO-R	amoA-2R'	amoA-2R	amoA-2RTG	amoA-2RTC	302R				
			New designation																														
			amoA21f	**amoA34f**	amoA40f	**amoA49f**	amoA60r	**amoA121f**	amoA151f	amoA154f	**amoA154fs**	amoA187f	amoA310f	amoA332f	amoA332fHY	amoA337p	**amoA359rC**	**amoA627r**	amoA665r	amoA680r	amoA681r	amoA686r	amoA820r	amoA822r	amoA822rTG	amoA822rTC	amoA828r				
Nm. europaea	*Nitrosomonas europaea*	L08050	0	2	0		3	0	1	2	1	0	4	1	1	0	1		0	2	1	0	0	0	0	1	0				
	Nitrosomonas eutropha	AY177932						2	1	2	1	2	4	0	1	4	2		1	3	3	2									
	Nitrosococcus mobilis	AF037108	0	2	0		3	0	1	2	1	0	4	1	1	0	1		0	2	1	0									
	Nitrosomonas sp. F3	AJ298691																	1	3	3	2									
	Nitrosomonas sp. F6	AJ298693																	1	3	3	2									
	Nitrosomonas sp. GH22	AF327917	1	2	0		4	2	1	2	1	2	4	0	1	4	2		2	3	3	2	2	1	1	2	1				
	Nitrosomonas sp. Nm93	AF272401																	2												
	Nitrosomonas sp. Nm103	AF272411																	0	2	1	0									
	Nitrosomonas sp. Nm104	AF272409																	2												
	Nitrosomonas sp. Nm107	AF272407																	2												
	Nitrosomonas sp. TK794	AB031869	1	2	0		4	1	1	2	1	2	4	0	1	4	2		2	3	3	2	2	1	1	2	1				
Nm. communis	*Nitrosomonas communis*	AF272399																	4	2		4									
	Nitrosomonas nitrosa	AF272404																5	2	4	4	4									
	Nitrosomonas sp. Nm33	AF272408																	2	3	3	3									
	Nitrosomonas sp. Nm41	AF272410																	1	2	3	4									
	Nitrosomonas sp. Nm58	AY123820																	2	2	4	4									
	Nitrosomonas sp. Nm148	AY123815																	2	4	4	4									
Nm. marina	*Nitrosomonas aestuarii*	AF272400																	2	1	3	3									
	Nitrosomonas marina	AF272405																	4	2	3	3									
	Nitrosomonas sp. C-113a	AF339042							0	1	0	2		1	2	2	0		4	3	2										
	Nitrosomonas sp. C-45	AF339041							0	1	0	1	3	1	0	2	0		3												
	Nitrosomonas sp. Nm51	AF272412																	4	3	3	2									
	Nitrosomonas sp. NO3W	AF339039							0	1	0	1	3	1	0	2	0		3	2	2										
	Nitrosomonas sp. TA-921-I-NH4	AF339043							0	2	0	1		1	2	2	1			2	2										
	Nitrosomonas sp. URW	AF339040							0	1	0	1	3	1	0	2	0		3												
Nm. oligotropha	*Nitrosomonas oligotropha*	AF272406																	2	4	2	2									
	Nitrosomonas ureae	AF272403																	4	4	4	4									
	Nitrosomonas sp. AL212	AF327918		3	0		5	4	1	2	1	2	4	1	1	4	2		4	2	3	3	2	3	3	2	3				
	Nitrosomonas sp. JL21	AF327919		2	0		4	2	1	3	1	1	4	2	1	5	2		3	3	1	1	2	3	3	2	3				
	Nitrosomonas sp. Nm143	AY123816																		2	2	2									
	Nitrosomonas sp. Nm47	AY123830																	3	4	3	3									
	Nitrosomonas sp. Nm59	AY123831																	2	4	3	4									
	Nitrosomonas sp. Nm84	AY123818																	2	3	1	1									
	Nitrosomonas sp. Nm86	AY123819																	2	3	2	2									
No cluster	*Nitrosomonas cryotolerans*	AF314753		4	3			0	0	0	0		4	0	1	2	2		4				2	2	2	2	4				
	Nitrosomonas halophila	AF272398																	3	4	4	4									
	Nitrosomonas halophila	AY026907																	4	4	4	4									
	Nitrosomonas oligotropha	AJ298709																	3	4	4	4									
Nc. oceani	*Nitrosococcus oceani*	AF047705				0			0	3	0		1					0		2	3										
	Nitrosococcus halophilus	AF272521											0					3		4	4										
	Nitrosococcus sp. C-113	AF153344				0			1	4	1		1					0		2											
MOB	*Methylocaldum gracile*	U89301							0	2	0		2																		
	Methylocaldum tepidum	U89304							0	2	0		3					1													
	Methylocapsa acidiphila	AJ278727											2																		
	Methylococcus capsulatus	L40804							0	2	0		3							2	1	3									
	Methylohalobius crimeensis	AJ581836											3					2													
	Methylomicrobium album	U31654											3					1													
	Methylomonas methanica	U31653											2					3													
	Methylosarcina lacus	AY007286							0	3	0		3					2													
	Methylothermus thermalis	AY829010											3																		
	Methylobacter sp. LW12	AY007285							0	3	0		2					2													

Sequences of beta-AOB were grouped in clusters according to their 16S rRNA phylogeny following the cluster designation of Purkhold *et al*. (2003). Sequences not grouped in any cluster are indicated as 'no cluster'. The number of mismatches is given in each box. Colour coding: grey = no sequence in this area; black = more than five mismatches; blue gradient = increasing weight of the mismatches (see methods) starting in 0 (white) to more than 4 (dark blue). For the explanation of new primer designation see Table 1 and text. Primers from this study are indicated in bold.

The primers 301F (amoA154f) and 302R (amoA828r) were designed as a primer pair to amplify a core region of 675 bp from *amoA* in 14 AOB (Norton *et al.*, 2002). The primer 301F (amoA154f) matches perfectly *Nitrosospira briensis*, *Nitrosovibrio tenuis*, *Nitrosospira* sp. 39-19 and *Nitrosomonas cryoloterans*, but has one to four mismatches with all other *Nitrosospira* and *Nitrosomonas* sequences, three to four mismatches with gammaproteobacterial AOB and two to three mismatches with *pmoA* (Table 2). The primer 302R (amoA828r) only targets *amoA* of gammaproteobacterial AOB, because its target region is deleted in the *amoA* of gammaproteobacterial AOB. Among betaproteobacterial AOB the primer 302R (amoA828r) matches perfectly only the sequences from *Nitrosovibrio tenuis* and *Nitrosomonas europaea*, but has one mismatch of low weight with *Nitrosospira* sp. Np 39–19, one to two mismatches of intermediate weight with *Nitrosospira briensis*, *Nitrosospira multiformis*, *Nitrosospira* sp. NpAV, *Nitrosomonas* sp. GH22 and *Nitrosomonas* sp. TK794, and three to four mismatches of high weight with *Nitrosomonas* sp. AL212, *Nitrosomonas* sp. JL21 and *Nitrosomonas cryotolerans*. Because of their length and base composition, both 301F (amoA154f) and 302R (amoA828r) have a very high T_m (Table 1), and therefore PCR conditions (for example salt and formamide concentration) have to be modified. Considering that the forward primer 301F (amoA154f) has potential to match simultaneously beta- and gammaproteobacterial AOB and MOB, the shorter version amoA154fs is suggested as a modification with lower T_m (Table 1) and significantly higher sequence similarity for all of the sequences (Table 2).

The primer amoA-1FF (amoA187f) (Hoshino *et al.*, 2001) was originally designed to amplify *Nitrosomonas europaea* in combination with the primer amoA-2R (amoA822r), for *in situ* PCR. In the sequence analysis, amoA-1FF (amoA187f) fully matches *Nitrosomonas europaea*, *Nitrosococcus mobilis* and *Nitrosospira* sp. NpAV, and has one to two mismatches with the other *Nitrosomonas* and *Nitrosospira* sequences (Table 2). The low number of mismatches with some sequences from other clusters of both lineages (*Nitrosovibrio tenuis*, *Nitrosospira* sp. Np 39-19, *Nitrosomonas* sp. C-113a and also uncultured clones) suggests that the amoA-1FF (amoA187f) is probably not specific for *Nitrosomonas europaea*.

The primer combination amoA-3F (amoA310f) and amoB-4R (amoB44r) was designed to amplify part of *amoA* from the gammaproteobacterial AOB *Nitrosococcus halophilus* (Purkhold *et al.*, 2000). amoa-3F (amoA310f) matches perfectly only the sequence from this species, has one mismatch with the two other gammaproteobacterial AOB, two or three mismatches with MOB and three to four mismatches of high weight with *Nitrosospira multiformis*, *Nitrosospira* sp. Np 39-19 and the majority of *Nitrosomonas* sequences (Table 2). According to the analysis with OLIGO (Table 1), highly stringent conditions are needed for reliable results with amoA-3F (amoA310f).

The primer pair amoA-1F (amoA332f) and amoA-2R (amoA822r) (Rotthauwe *et al.*, 1997) is the most widely used to amplify *amoA* in environmental studies, despite the differences in the T_m between the primers (Table 1). amoA-1F (amoA332f) is located in a region conserved in all betaproteobacterial AOB; it matches perfectly or with one to two mismatched sequences from betaproteobacterial AOB, but it does not match sequences from gammaproteobacterial AOB. The primer amoA1F mod (amoA332fHY), which is a modified version including two wobble positions to increase sequence identity with cultured betaproteobacterial AOB (Stephen *et al.*, 1999), matched the same spectrum of sequences, but produced differences in the weight of the mismatches (Table 2) and T_m (Table 1). The primer amoA-2R (amoA822r) matched only sequences from betaproteobacterial AOB, but had lower specificity than 302R (amoA828r) (Norton *et al.*, 2002). Several variants of amoA-2R (amoA822r) have been proposed, including the primer amoA-2R′ (amoA820r) (Okano *et al.*, 2004), a shorter version with lower sequence similarity to the target region and additional unspecific matches in other regions of *amoA* from both cultured and uncultured species. Other variants of amoA-2R (amoA822r) have been proposed specifically for denaturing gradient gel electrophoresis (DGGE), in order to reduce the number of wobble positions that usually generate double bands in denaturing gels. These include amoA-2R-TC (amoA822rTC) (Nicolaisen & Ramsing, 2002) or amoA-2R-TG (amoA822rTG) (Okano *et al.*, 2004). These primers matched the same sequences as the original version but showed differences in the weight of the mismatches (Table 2) and higher T_m (Table 1).

In addition to the primers described to amplify *amoA*, the probe A337 (amoA337p) (Okano *et al.*, 2004) has been published for FISH. Although this probe has, in most of the cases, mismatches with sequences from cultures of all betaproteobacterial AOB clusters, it has fewer than five mismatches with all sequences from cultured betaproteobacterial AOB and all sequences from uncultured clones, suggesting that it is located in a region suitable for the design of a general primer for detection of betaproteobacterial AOB.

Sequence analysis of primers for simultaneous detection of *amoA* and *pmoA*

The common evolutionary origin of AMO and particulate methane monooxygenase (pMMO) (Holmes *et al.*, 1995) suggests the possibility of finding conserved regions for designing primers that amplify both genes. The primer pair A189 (amoA151f) and A682 (amoA681r) was used for this purpose (Holmes *et al.*, 1995). A189 (amoA151f) is located in the same conserved region as 301F (amoA154f) (Norton

et al., 2002) and amoA154fs. It has a perfect match with the majority of sequences from beta- and gammaproteobacterial AOB and MOB (Table 2). The reverse primer A682 (amoA681r) matches perfectly only sequences from *Nitrosospira* clusters 2 and 0. A further modification of this primer, A682 mod (amoA680r) (Nold *et al.*, 2000), was designed to increase the sensitivity for gammaproteobacterial AOB. However, as shown in Table 2, the matches with cultured AOB improved only slightly.

Sequence analysis of *amoC* and *amoB* primers

Both *amoB* and *amoC* are likely to be good alternatives as functional markers for molecular studies on AOB because, they code for essential parts of the multi-subunit AMO enzyme, which may be involved in the active site by extrapolation with the homologous *pmoC* and *pmoB* (Lieberman & Rosenzweig, 2005; Balasubramanian & Rosenzweig, 2007), and have a suitable size for phylogenetic inferences (*amoC* has around 800 bp and *amoB* is the longest of the three genes with more than 1200 bp). However, compared with *amoA*, *amoB* and *amoC* have been neglected despite the their potential for additional sequence information.

Consequently, only a few primers have been described to amplify these genes. Primer 305F (amoC763f) (Norton *et al.*, 2002) was designed to be used in combination with 304R (amoA60r) to generate a PCR product encompassing the 3′ end of *amoC*, the intergenic region with *amoA* and the 5′ part of *amoA* (see Fig. 1). Alignment with *amoC* sequences showed that primer 305F (amoC763f) does not match perfectly any of the sequences analyzed (Fig. 2) and possesses a significant difference in T_m (Table 1) with 304R (amoA60r). The primer 305F (amoC763f) has between one and six mismatches with betaproteobacterial AOB and more than 10 mismatches with gammaproteobacterial AOB and

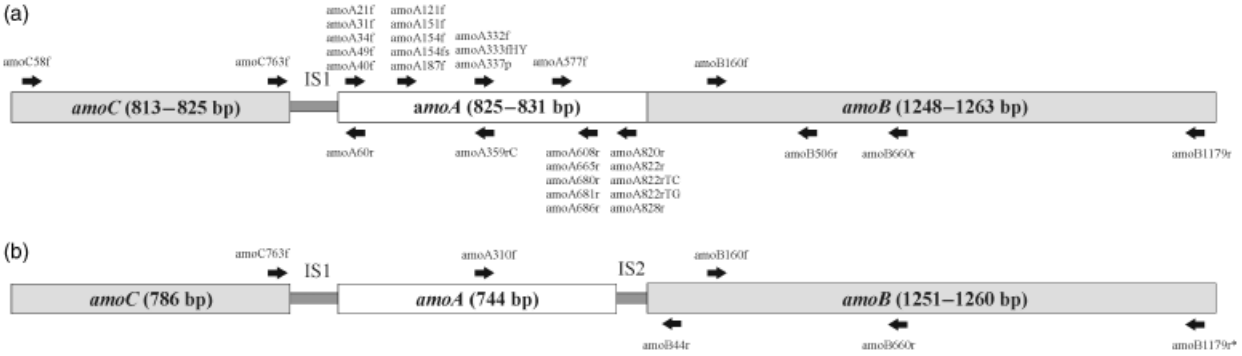

Fig. 1. Schematic diagram of the *amoCAB* operon in beta- (a) and gamma-AOB (b). IS, intergenic regions. The position and orientation of the different primers are shown by arrows. For primer designation see Table 1.

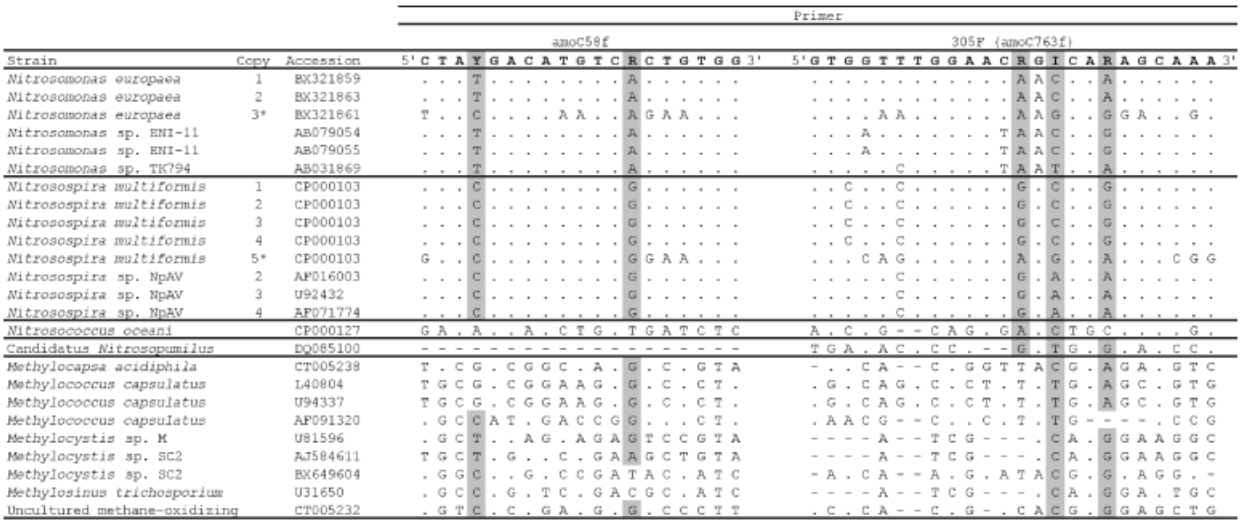

Fig. 2. Alignment using CLUSTALW of *amoC* primers with all sequences available. Matches with the primer sequences are indicated by dots. Matches in wobble positions are shown as shaded. The asterisk denotes the *amoC* copies of *Nitrosomonas europaea* and *Nitrosospira multiformis* not belonging to the *amoCAB* operon.

MOB. The two copies of *amoC* that are not located in the *amoCAB* operon of betaproteobacterial AOB had more mismatches at different positions with the primers (Fig. 2), suggesting that new primers can be designed to target these singleton copies specifically.

The primer amoB-4R (amoB44r) (Purkhold *et al*., 2000), which was designed to amplify *amoAB* from *Nitrosococcus halophilus* in combination with the primer amoA-3F (amoA 310f), does not match perfectly any sequence analyzed (Fig. 3). This region is not highly conserved either in gamma- or in betaproteobacterial AOB.

The primer pair amoBMf (amoB160f) and amoBMr (amoB660r) (Calvo & Garcia-Gil, 2004) has been published recently in order to use *amoB* as an alternative molecular

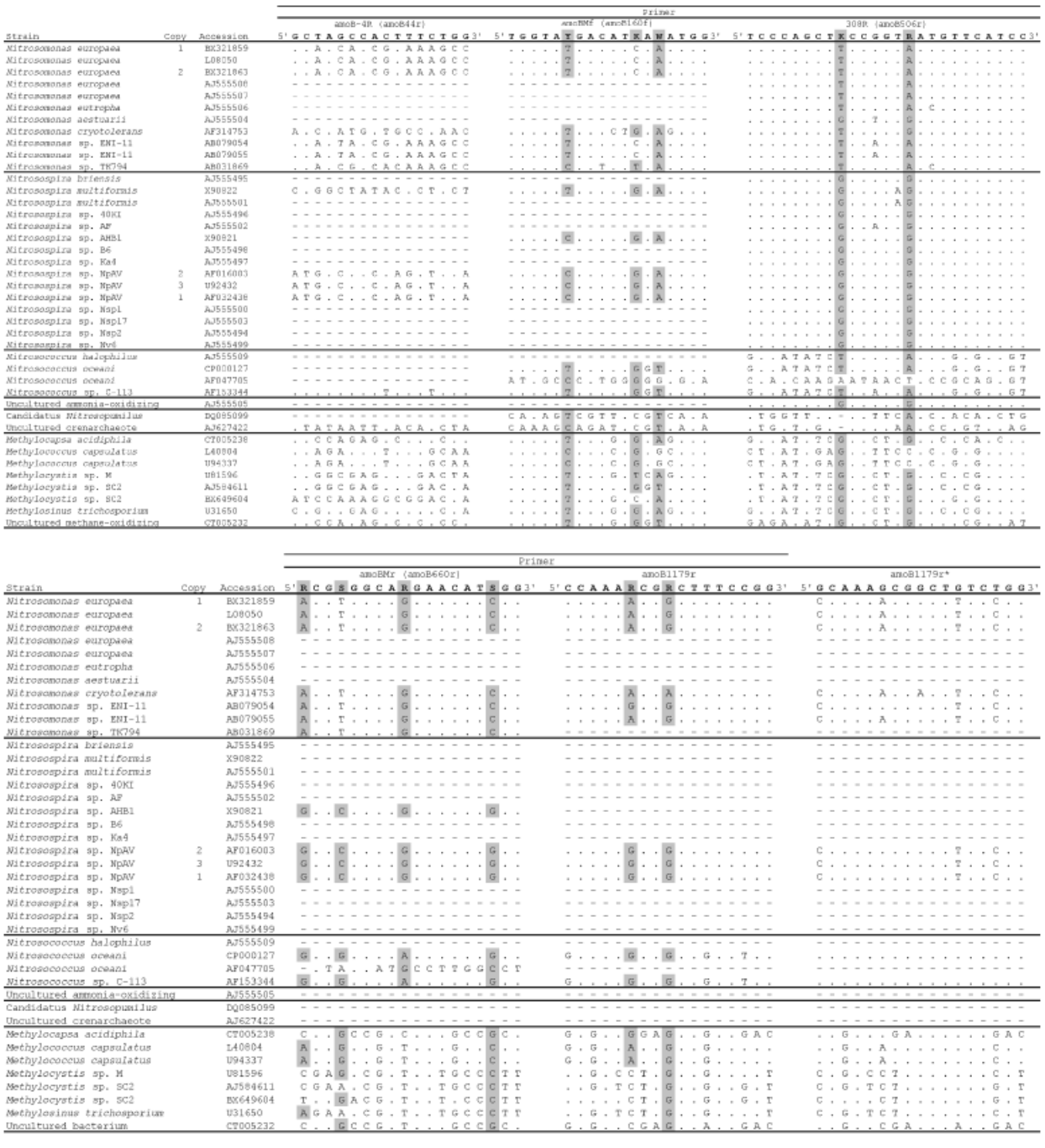

Fig. 3. Alignment using CLUSTALW of *amoB* primers with all sequences available. Matches with the primer sequences are indicated by dots. Matches in wobble positions are shown as shaded. Dashes represent gaps in the alignment.

marker for AOB. Both primers target regions relatively conserved in beta- and some gammaproteobacterial AOB (Fig. 3), but so far they have not been used extensively in environmental samples. The annealing temperature suggested for this primer pair (Calvo & Garcia-Gil, 2004) is significantly higher than the calculated values (Table 1).

The primer 308R (amoB506r) (Norton *et al.*, 2002) was proposed to be combined with 305F (amoC763f) as an alternative to obtain the full length of the *amoA* gene and its flanking regions. In the alignment with *amoB* sequences (Fig. 3), this primer had 10–11 mismatches with sequences from gammaproteobacterial AOB and is therefore probably suitable only for betaproteobacterial AOB.

Very recently, the *amoB* sequences from two Archaea have been deposited in GenBank (Konneke *et al.*, 2005; Treusch *et al.*, 2005). These partial sequences were too short for sequence comparison with the majority of primers analyzed here. The primers amoBMf (amoB160f) and 308R (amoB506r) presented more than 12 mismatches and are not expected to target these sequences.

Description of new primers for amplification of the *amoCAB* operon

To examine the possibility of amplifying the almost complete *amoCAB* operon, sequence conservation was inspected in the few sequences available for the flanking genes *amoC* and *amoB*. The primers amoC58f and amoB1179r (Table 1) were designed to amplify the largest segment possible of the operon, which includes the three genes and the intergenic regions. The size of the PCR product is variable due to differences in the length of the genes and especially of the intergenic regions, but should be around 2900 bp. Matching of the primer amoC58f with the *amoC* sequences available in GenBank is shown in Fig. 2. A BLAST search retrieved only sequences from betaproteobacterial AOB and did not have any unspecific match. This primer matched perfectly the sequences from betaproteobacterial AOB, except for the *amoC* copies of *Nitrosomonas europaea* and *Nitrosospira multiformis* that are not located in an operon. These extra copies of *amoC* are expected to be excluded from the amplification because of the difference in the sequence but also the use of the reverse primer amoB1179r, which is located at the end of the *amoB* gene. The primer amoB1179r matches in a highly conserved region of *amoB* from betaproteobacterial AOB and *Nitrosococcus halophilus* (Fig. 3). In a BLAST search, it matched all *amoB* from betaproteobacterial AOB. In a modification of this primer (amoB1179rGam) the specificity is shifted to target only gammaproteobacterial AOB.

The application of *amoA* for phylogenetic inference is partially limited due to short length and high conservation of the fragment analyzed (Purkhold *et al.*, 2003). Therefore, one of the main challenges for applying this gene as a functional molecular marker is the search of primers that allow the amplification of a longer *amoA* fragment. Different conserved positions were detected in the *amoA* alignment. The primer amoA34f was designed to target positions close to the 5′ region of the gene that can be used in combination with primers for the 3′ region of the gene such as amoA-2R (Rotthauwe *et al.*, 1997) or 302R (Norton *et al.*, 2002) to amplify almost the whole of *amoA*. This primer retrieved sequences from all beta AOB included in this study (Table 2), and has been already used to characterize AOB communities in marine environments (Molina *et al.*, 2007). The wider spectrum of betaproteobacterial AOB recognized by the primer amoA34f, compared with the primer amoAF (Sinigalliano *et al.*, 1995), makes amoA34f a better option for PCR in environments not dominated by *Nitrosomonas*-like AOB.

The primers amoA121f and amoA359rC were designed to amplify an internal fragment from betaproteobacterial AOB suitable for DGGE. The primer amoA121f matches perfectly all *Nitrosospira* spp. and some *Nitrosomonas* spp. and with one to four mismatches *Nitrosomonas eutropha, Nitrosomonas* sp. GH22, *Nitrosomonas* sp. TK794, *Nitrosomonas* sp. AL212, *Nitrosomonas* sp. JL21. It has more than five mismatches with sequences from gammaproteobacterial AOB (Table 2). The reverse primer amoA359rC, having nine bases overlap with amoA-1F (amoA332f) (Rotthauwe *et al.*, 1997), matches perfectly the sequences from all *Nitrosospira* clusters and displays high similarity with the *Nitrosomonas* clusters. A former version of the primer combination amoA121fgc-amoA359r, which was originally designated amoA-3F/amoA-4R, designed in the laboratory was previously used by other research groups to analyze the impact of soil management on the diversity of AOB in soil (Webster *et al.*, 2002). The primer amoA359rC reported in this manuscript is an improved variant of the original primer designated amoA4-R, which was used in the DGGE without wobble positions to avoid artifacts. Besides the use of this primer combination for DGGE, the size of the expected PCR product makes it also potentially useful for quantification of AOB by real-time PCR.

Although the number of *amoA* sequences from gammaproteobacterial AOB is very limited (only two complete sequences), the primer pair amoA49f and amoA627r was designed to tentatively amplify a fragment of 559 bp exclusively from gammaproteobacterial AOB. These primers, when checked in GenBank by BLAST, matched only the sequences used for primer design. Similarly, the evaluation in ARB showed that the forward primer matches only the two gammaproteobacterial AOB while the reverse primer has three mismatches with *Nitrosococcus halophilus* but no mismatches with *Nitrosococcus oceani*. Between one and three mismatches were recorded with some MOB and more

than five with all betaproteobacterial AOB (Table 2). These primers have a similar melting temperature (Table 1), desirable for specific amplification.

Conclusion

The re-examination of specific primers to amplify the *amoCAB* operon carried out in this study by sequence analyses indicates possible strength and weakness of primers to study community composition of AOB in environmental samples. The use of new primers targeting new regions in the complete operon can contribute to the information on the evolution and function of the *amoCAB* operon in AOB. Additionally, nested amplification offers the possibility of increasing PCR sensitivity for AOB detection in environmental samples.

Acknowledgements

This research was partially supported by G.I.F. (German–Israel Foundation) grant no. I-711-83.8/2001 and BSF (Binational Science Foundation) grant no. 2002-206. The authors wish to thank the Max-Planck Society and the G.I.F. for financial support of P. J. and O.-S.K. during this study.

Authors' contribution

P.J. and O.-S.K. contributed equally to this paper.

References

Aakra A, Utaker JB & Nes IF (2001) Comparative phylogeny of the ammonia monooxygenase subunit A and 16S rRNA genes of ammonia-oxidizing bacteria. *FEMS Microbiol Lett* **205**: 237–242.

Alzerreca JJ, Norton JM & Klotz MG (1999) The *amo* operon in marine, ammonia-oxidizing gamma-proteobacteria. *FEMS Microbiol Lett* **180**: 21–29.

Balasubramanian R & Rosenzweig AC (2007) Structural and mechanistic insights into methane oxidation by particulate methane monooxygenase. *Acc Chem Res* **40**: 573–580.

Bothe H, Jost G, Schloter M, Ward BB & Witzel K-P (2000) Molecular analysis of ammonia oxidation and denitrification in natural environments. *FEMS Microbiol Rev* **24**: 673–690.

Calvo L & Garcia-Gil LJ (2004) Use of *amoB* as a new molecular marker for ammonia-oxidizing bacteria. *J Microbiol Methods* **57**: 69–78.

Chain P, Lamerdin J, Larimer F *et al.* (2003) Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea*. *J Bacteriol* **185**: 2759–2773.

Head IM, Hiorns WD, Embley TM, McCarthy AJ & Saunders JR (1993) The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. *J Gen Microbiol* **139**: 1147–1153.

Holmes AJ, Costello A, Lidstrom ME & Murrell JC (1995) Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionary related. *FEMS Microbiol Lett* **132**: 203–208.

Hoshino T, Noda N, Tsuneda S, Hirata A & Inamori Y (2001) Direct detection by *in situ* PCR of the *amoA* gene in biofilm resulting from a nitrogen removal process. *Appl Environ Microbiol* **67**: 5261–5266.

Hyman MR & Arp DJ (1992) $^{14}C_2H_2$- and $^{14}CO_2$-labeling studies of the de novo synthesis of polypeptides by *Nitrosomonas europaea* during recovery from acetylene and light inactivation of ammonia monooxygenase. *J Biol Chem* **267**: 1534–1545.

Juretschko S, Timmermann G, Schmid M, Schleifer KH, Pommerening-Röser A, Koops HP & Wagner M (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.

Klotz MG, Arp DJ, Chain PS *et al.* (2006) Complete genome sequence of the marine, chemolithoautotrophic, ammonia-oxidizing bacterium *Nitrosococcus oceani* ATCC 19707. *Appl Environ Microbiol* **72**: 6299–6315.

Konneke M, Bernhard AE, de la Torre JR, Walker CB, Waterbury JB & Stahl DA (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* **437**: 543–546.

Kowalchuk GA & Stephen JR (2001) Ammonia-oxidizing bacteria: a model for molecular microbial ecology. *Annu Rev Microbiol* **55**: 485–529.

Lieberman RL & Rosenzweig AC (2005) The quest for the particulate methane monooxygenase active site. *Dalton Trans* **7**: 3390–3396.

Ludwig W, Strunk O, Westram R *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363–1371.

McTavish H, Fuchs JA & Hooper AB (1993) Sequence of the gene coding for ammonia monooxygenase in *Nitrosomonas europaea*. *J Bacteriol* **175**: 2436–2444.

Mobarry BK, Wagner M, Urbain V, Rittmann BE & Stahl DA (1996) Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Appl Environ Microbiol* **62**: 2156–2162.

Molina V, Ulloa O, Farías L, Urrutia H, Ramírez S, Junier P & Witzel KP (2007) Ammonia-oxidizing beta-proteobacteria from the oxygen minimum zone off northern Chile. *Appl Environ Microbiol* **73**: 3547–3555.

Nicolaisen MH & Ramsing NB (2002) Denaturing gradient gel electrophoresis, (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. *J Microbiol Methods* **50**: 189–203.

Nold SC, Zhou J, Devol AH & Tiedje JM (2000) Pacific Northwest marine sediments contain ammonia-oxidizing bacteria in the beta subdivision of the proteobacteria. *Appl Environ Microbiol* **66**: 4532–4535.

Norton JM, Alzerreca JJ, Suwa Y & Klotz MG (2002) Diversity of ammonia monooxygenase operon in autotrophic ammonia-oxidizing bacteria. *Arch Microbiol* **177**: 139–149.

Okano Y, Hristova KR, Leutenegger CM *et al.* (2004) Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. *Appl Environ Microbiol* **70**: 1008–1016.

Prosser JI (1989) Autotrophic nitrification in bacteria. *Adv Microb Physiol* **30**: 125–181.

Purkhold U, Pommerening-Röser A, Juretschko S, Schmid MC, Koops HP & Wagner M (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys. *Appl Environ Microbiol* **66**: 5368–5382.

Purkhold U, Wagner M, Timmermann G, Pommerening-Röser A & Koops HP (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.

Rotthauwe JH, Witzel K-P & Liesack W (1997) The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl Environ Microbiol* **63**: 4704–4712.

Sinigalliano CD, Kuhn DN & Jones RD (1995) Amplification of the *amoA* gene from diverse species of ammonium-oxidizing bacteria and from an indigenous bacterial population from seawater. *Appl Environ Microbiol* **61**: 2702–2706.

Stephen JR, Chang YJ, Macnaughton SJ, Kowalchuk GA, Leung KT, Flemming CA & White DC (1999) Effect of toxic metals on indigenous soil beta-subgroup proteobacterium ammonia oxidizer community structure and protection against toxicity by inoculated metal-resistant bacteria. *Appl Environ Microbiol* **65**: 95–101.

Treusch AH, Leininger S, Kletzin A, Schuster SC, Klenk HP & Schleper C (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.

Wagner M, Amann R, Lemmer H & Schleifer KH (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.

Wagner M, Rath G, Amman R, Koops H-P & Schleifer KH (1995) *In situ* identification of ammonia-oxidizing bacteria. *Syst Appl Microbiol* **18**: 251–264.

Webster G, Embley TM & Prosser JI (2002) Grassland management regimens reduce small-scale heterogeneity and species diversity of beta-proteobacterial ammonia oxidizer populations. *Appl Environ Microbiol* **68**: 20–30.