Community analysis of betaproteobacterial ammonia-oxidizing bacteria using the amoCAB operon

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Abstract The genes and intergenic regions of the amoCAB operon were analyzed to establish their potential as molecular markers for analyzing ammonia-oxidizing betaproteobacterial (beta-AOB) communities. Initially, sequence similarity for related taxa, evolutionary rates from linear regressions, and the presence of conserved and variable regions were analyzed for all available sequences of the complete amoCAB operon. The gene amoB showed the highest sequence variability of the three amo genes, suggesting that it might be a better molecular marker than the most frequently used amoA to resolve closely related AOB species. To test the suitability of using the amoCAB genes for community studies, a strategy involving nested PCR was employed. Primers to amplify the whole amoCAB operon and each individual gene were tested. The specificity of the products generated was analyzed by denaturing gradient gel electrophoresis, cloning, and sequencing. The fragments obtained showed different grades of sequence identity to amoCAB sequences in the GenBank database. The nested PCR approach provides a possibility to increase the sensitivity of detection of amo genes in samples with low abundance of AOB. It also allows the amplification of the almost complete amoA gene, with about 300 bp more sequence information than the previous approaches. The coupled study of all three amo genes and the intergenic spacer regions that are under different selection pressure might allow a more detailed analysis of the evolutionary processes, which are responsible for the differentiation of AOB communities in different habitats.

Keywords Ammonia-oxidizing betaproteobacteria · PCR primers · amoC · amoA · amoB
Introduction

Ammonia oxidation to nitrite, the first step in nitrification, is primarily mediated by chemolithoautotrophic microorganisms belonging to beta- and gammaproteobacteria (Prosser 1989; Head et al. 1993; Teske et al. 1994; Kowalchuk and Stephen 2001) and archaea (Konneke et al. 2005; Treusch et al. 2005). Ammonia-oxidizing bacteria (AOB) are difficult to culture, and therefore, several molecular techniques, including the analysis of the 16S rRNA gene, have been developed to study their diversity in the environment without cultivation (Bothe et al. 2000; Kowalchuk and Stephen 2001; Prosser and Embley 2002).

Although the 16S rRNA gene is a good phylogenetic marker, it is not necessarily related to the physiology of the target organisms (Kowalchuk and Stephen 2001; Calvo and Garcia-Gil 2004), and a slight lack of specificity may shift the spectrum of sequences amplified by PCR toward phylogenetically related but physiologically and ecologically different organisms (Bothe et al. 2000; Junier et al. 2008a, b). Therefore, functional markers such as the genes encoding for key enzymes involved in ammonia oxidation provide an alternative in ecological studies (Rothhauwe et al. 1997). Particularly in the case of AOB, one of these genes, amoA, has been traditionally used to study AOB in environmental samples (Rothhauwe et al. 1997; Alzerreca et al. 1999; Aakra et al. 2001a, b; Norton et al. 2002).

The gene amoA, together with the genes amoC and amoB, codify for the different subunits of the ammonia monooxygenase (AMO). The AMO is a membrane-bound, multiple-subunit enzyme responsible for the oxidation of ammonia to hydroxylamine, which is further converted to nitrite by the hydroxylamine oxidoreductase (Hyman and Arp 1992). In AOB, these genes are organized in the amoCAB operon (Norton et al. 2002). Homologs of amoA, B, and C were found in archaea (Konneke et al. 2005; Treusch et al. 2005), but these genes have low similarity to their bacterial counterparts.

Despite the potential of the amoCAB genes, the portion of the gene amoA generally used as a molecular marker to study the diversity of AOB (Rothhauwe et al. 1997) is relatively short and highly conserved, providing less resolution than the 16S rRNA gene (Koops et al. 2003; Purkhold et al. 2003). Therefore, it seemed advantageous to have alternative targets that could offer additional information on the diversity, evolution, and function of this group in the environment. All the genes on the amoCAB operon offer this possibility. For example, amoC and amoB can be alternative markers for molecular studies of AOB, considering their size (amoC is 800 bp, and amoB, the longest of the three genes, is around 1,200 bp) and the functional role of the protein subunits coded by amoC and amoB.

In this study, we have analyzed the suitability of amoC, amoB, and almost the whole of amoA as molecular markers for AOB. Initially, sequence analysis was carried out with sequences from cultured strains of AOB and related taxa to evaluate the phylogenetic relationships, the evolutionary rates, and the presence of conserved or variable regions in the amoCAB genes. Based on these results, a strategy was developed allowing the retrieval and analysis of amoCAB sequences from environmental samples. The feasibility of a nested approach to amplify the amoCAB genes was tested. The results demonstrate the high potential of the amoCAB genes for studying AOB in the environment and the feasibility of a nested PCR approach to amplify environmental sequences of the three genes.

Materials and methods

Sequences and phylogenetic analysis

All amoCAB sequences deposited in GenBank, as well as the sequences from the clones of this study, were integrated into the phylogenetic software ARB (Ludwig et al. 2004). Phylogenies were calculated based on alignments of nucleotide and deduced amino acid sequences using the PHYLIP program distmat from EMBoss (Rice et al. 2000), using the Tamura correction method. Similarity was calculated based on the genetic distance. For those species for which the complete sequence of all amoCAB genes (Nitrosonomas europaea, Nitrosomonas sp. TK794, Nitrosomonas sp. EN111, Nitrosospira sp. NpAV, Nitrosospiro multiformis, and Nitrosococcus oceanii) or pmoCAB (Methylcococcus capsulatus, Methylcystis sp. M, Methylcystis sp. SC2, and Methylisnus trichosporium) was available, the similarity values were plotted using in each axis the similarity for one of the genes. Correlation plots between amoA/amoC, amoA/amoB, amoC/amoB, and their respective pmoCAB were calculated in this way. Plots of sequence similarity along the alignment were calculated using the program plotcon from EMBoss (Rice et al. 2000).

Samples and DNA extraction

The following strains of beta-AOB were used as positive controls for PCR: N. europaea ATCC 19718, Nitrosonomas eutropha C-71, Nitrospira briensis C-128, Nitrospira tenuis Nv1, Nitrosomonas sp. L13. In order to test the specificity of the primers for beta-AOB, the following negative controls were also assayed: N. oceanii ATCC 19707 (gamma-AOB) and the type I and II methane-
oxidizing bacteria (MOB) strains *M. capsulatus* Bath NCIMB 11853 and *M. parvus* OBBP. Samples were obtained from rhizospheric soil of pea cultures in northern Germany, the Jordan River (Israel), Lake Schöhsee (Germany), Lake Plußsee (Germany), and the water column and water–sediment interface at Boknis Eck out of the Kiel Fjord at the German coast of the Baltic Sea. For DNA extraction, water samples were filtered through 0.2-μm pore size filters (Supor-200, PALL Life Sciences) and frozen at −18°C. DNA was extracted using the UltraClean Soil DNA kit (MoBio), following the manufacturer’s guidelines. Concentration and quality of the DNA were checked by electrophoresis on 0.8% agarose gels stained with ethidium bromide.

**PCR**

Several combinations of PCR primers (Holmes et al. 1995; Rotthauwe et al. 1997; Norton et al. 2002; Calvo and Garcia-Gil 2004; Junier et al. 2008a, b) were used to amplify different regions of the *amoCAB* operon in beta-AOB (Table 1). For amplification of the almost complete *amoCAB* operon, the forward primer amoC58f and the reverse primer amoB1179r (Table 1) were used. PCR was carried out with the Expand High Fidelity PCR system (Roche) in 25 μl containing 1× PCR buffer, 200 μM of each dNTP, 200 mM of each primer, 2.5 mM MgCl₂, 1% formamide, 0.1% BSA, and 2.6 U of enzyme mix. The temperature program consisted of 94°C for 2 min and 30 cycles of 94°C for 15 s, 56°C for 1 min, and 68°C for 5 min. The *amoCAB* products (approximately 3,000 bp) were purified to remove primer excess (Multiscreen, Millipore), resuspended in HPLC water, and used as template for further nested amplifications indicated in Fig. 1. Nested PCR was carried out as above, but 1 U of Taq DNA polymerase (Roche) was used instead of the Expand High Fidelity PCR system. The temperature program consisted of initial denaturation at 94°C for 5 min, 25 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1.5 min.

**DGGE**

For denaturing gradient gel electrophoresis (DGGE) of *amoA*, the *amoCAB* products were re-amplified with the primer pair amoA121f with a 40 bp GC-clamp attached (Muyzer et al. 1993) and amoA359rC in a touchdown temperature program with annealing temperatures decreasing from 65°C to 55°C (Muyzer et al. 1993). DGGE was performed according to the manufacturer’s guidelines with the D-Gene System (BioRad) in polyacrylamide gels (7.5% of 37:1 acrylamide–bisacrylamide mixture in 0.5× TAE buffer, 0.75 mm thick, 16×10 cm) with a gradient of 35% to 75% of denaturants (100% denaturants contained 420 g/l urea and 400 ml/l deionized formamide). Gels were run in 0.5× TAE buffer at 200 V and constant temperature of 60°C for 10 h. To recover the DGGE bands for sequencing, DGGE gels were stained with SYBR gold (Molecular Probes). The most prominent bands were punched with a sterile pipette tip, transferred to 100 μl HPLC water, and maintained overnight at 4°C for DNA diffusion. The eluted DNA was used as template for PCR with the primers amoA121f/amoA359rC, under the conditions described above. The PCR products were purified with a Multiscreen plate (Millipore) and used for templates for sequencing with the primer amoA359rC. The BigDye Terminator Cycle Sequencing Kit v.1.1 (Applied Biosystems), recommended

### Table 1

| Primer  | Position | Sequence 5′–3′ | Reference |
|---------|----------|----------------|-----------|
| amoC58f | 58–72    | CTA YGA CAT GTC RCT GTG G | Junier et al. 2008a, b |
| 305F    | 763–786  | GTG GTT TGG AAC RGI CAR AGC AAA | Norton et al. 2002 |
| amoA34f | 34–57    | GCG GCR AAA ATG CCG CCG GAA GCG | Molina et al. 2007 |
| amoA121f| 121–137  | ACC TAC CAC ATG CAC TT | Junier et al. 2008a, b |
| amoA-1F | 332–349  | GGG GTT TCT ACT GGT GGT | Rotthauwe et al. 1997 |
| amoA349r| 332–349  | ACC ACC AGT AGA AAC CCC | This study |
| amoA359rC| 340–359 | GGG TAG TGC GAC CAC CAG TA | Junier et al. 2008a, b |
| amoA664f| 664–681  | GCS TTC TTC TNC GCS TTC | This study |
| A682    | 664–681  | GAA SGC NGA GAA GAA SGC | Holmes et al. 1995 |
| amoA802f| 802–822  | GAA GAA GGC TTT SCM GAG GGG | This study |
| amoA-2R | 802–822  | CCC CTC KGS AAA GCC TTC TTC | Rotthauwe et al. 1997 |
| amoBMf  | 160–177  | TGG TAY GAC ATK AWA TGG | Calvo and Garcia-Gil 2004 |
| amoBMr  | 643–660  | RCG SGG CAR GAA CAT SGG | Calvo and Garcia-Gil 2004 |
| amoB1179r| 1164–1179| CCA AAR CGR CTT TCC GG | Junier et al. 2008a, b |
Fig. 1 Phylogenetic tree based on AmoC (a), AmoA (b), and AmoB (c) sequences using all complete sequences of cultured representatives of ammonia-oxidizing bacteria (AOB). Sequences of the related PmoC, PmoA, and PmoB from methane-oxidizing bacteria (MOB) were also included. Phylogenies were constructed with the PHYLIP subroutine by Fitch-Margoliash in ARB. Bootstraps values are indicated. Asterisk AmoC copies outside the amoCAB operon in N. europaea ATCC 19718 and N. multiformis ATCC 25196

Nitrosospirota

Nitrosomonas

β-AOB

γ-MOB (pmoC)

α-MOB (pmoC)

Nitrosospirota

Nitrosomonas

β-AOB

γ-MOB (pmoA)

β-AOB

γ-MOB (pmoA)

γ-MOB (pmoB)

γ-MOB (pmoB)

β-AOB

β-AOB

Nitrosospirota

Nitrosomonas

β-AOB

γ-MOB (pmoB)

γ-MOB (pmoB)
for short readings, was used. Sequences were analyzed in an ABI3100 automated sequencer (Applied Biosystems). BLAST search (Altschul et al. 1997) was used to determine similarity with sequences in the GenBank database.

Cloning and sequencing

Pfu DNA polymerase (Promega) was used to minimize PCR errors in the amplification of products for cloning. Triplicate PCR reactions were combined and concentrated in a Multiscreen plate (Millipore) for cloning with the Zero Blunt PCR Cloning Kit (Invitrogen) according to the manufacturer's guidelines. Twelve clones were picked and checked for inserts of the appropriate size by PCR with the vector-specific primers M13F/M13R. Sequencing of both strands was performed with M13F and M13R primers using the BigDye Terminator Cycle Sequencing Kit v3.1 and ABI 3100 capillary sequencer (Applied Biosystems). The sequences were checked using BLASTX (Altschul et al. 1997). Sequences from this study have been deposited in GenBank under the accession numbers EF204958–EF204996 and EF219168–EF219191.

Results

AOB phylogeny inferred from amoC, amoA, and amoB sequences

Sequences from the complete amoC, amoA, and amoB genes were used to reconstruct the phylogenetic relationships of culture strains of AOB. Additionally, sequences from the evolutionary equivalent pmoCAB genes, which code for the particulate methane monooxygenase in MOB, were included in the analysis.

In the phylogenetic trees from the deduced amino acidic AmoA/PmoA, AmoB/PmoB, and AmoC/PmoC sequences, AmoCAB sequences from betaproteobacterial AOB (comprising the Nitrosospira and Nitrosomonas lineages) always formed a cluster separated from gammaproteobacterial AOB and PmoCAB in MOB (Fig. 1). The separation of the betaproteobacterial AOB from the other sequences was supported by bootstrap values of 91% (AmoA), 87% (AmoC), and 60% (AmoB). Sequences from betaproteobacterial AOB were further separated into two groups, one corresponding to the Nitrosomasia lineage (including Nitrosomasia mobilis) and the second to the Nitrosospira lineage (Fig. 1). The separation of the sequences from the two lineages of betaproteobacterial AOB (Nitrosospira and Nitrosomonas) was supported by high bootstrap values for all the three genes (94–99%).

Single amoC copies that are located elsewhere in the complete genome sequence of N. europaea ATCC 19718 (Chain et al. 2003) and N. multiformis ATCC 25196 were also included in the analysis. One of the two additional AmoC copies in N. multiformis ATCC 25196 and the only one of N. europaea ATCC 19718 clustered together with other beta-AOB AmoC (Fig. 1a). However, they formed an independent cluster separated from the other AmoC copies in these species.

Co-evolution of the different genes in the amoCAB operon

To establish whether the evolution rate of individual amoCAB genes was correlated, the sequence similarity derived from evolutionary distance between pairs of species was analyzed. Sequence similarity values were represented in correlation plots (Fig. 2) in which each axis represents the distance for one of the genes (e.g., amoA in axis X versus amoB or amoC in axis Y). A linear regression was applied to the plots. A high r2 value will be an indication of co-evolution between pairs of genes, while the slope of the curve will indicate if, despite being co-evolving, one of the genes evolves faster than the other. This analysis was carried out with species for which the complete sequence of all amoCAB genes (N. europaea, Nitrosomonas sp. TK794, Nitrosomonas sp. EN11, Nitrosospira sp. NpAV, N. multiformis, and N. oceani) and pmoCAB (M. capsulatus, Methylocystis sp. M, Methylocystis sp. SC2, and M. trichosporium) were available. A significant linear correlation was obtained for the comparisons of amoA/pmoA versus amoB/pmoB (r2=0.96; Fig. 2a), amoA/pmoA versus amoC/pmoC (r2=0.95; Fig. 2c), and amoC/pmoC versus amoB/pmoB (r2=0.95; Fig. 2e), suggesting that the genes encoding the different subunits of the AMO and pMMO are co-evolving. The same analysis was conducted only with the amoCAB sequences of beta-AOB (Fig. 2b, d, and f), obtaining even higher r2 values (amoA/amoB=0.98; amoA/amoC=0.98; amoC/amoB=0.97), confirming the co-evolution of the different genes in the amoCAB operon.

Detection of conserved and variable regions in the amoCAB operon

The average similarity values obtained for the full-length amoCAB genes for the different lineages of beta- and gamma-AOB were calculated (Table 2). In beta-AOB, the lowest similarity values were obtained for amoB, followed by amoA and amoC. However, within the Nitrosomonas lineage, amoA is considerably more variable than amoC and amoB, while in Nitrosospira, amoA and amoB are both more variable than amoC. Sequence similarity of the extra amoC copies of N. multiformis ATCC 25196 and of N. europaea ATCC 19718 with other amoC sequences from beta-AOB is very low (66%). Sequence similarity was higher in gamma-AOB for the three genes compared to...
beta-AOB although this could be an artifact due to the smaller number of complete \textit{amoCAB} sequences from different \textit{Nitrosococcus} species.

Local changes in sequence conservation were inspected throughout the alignments to identify regions with less conservation in the different genes of the \textit{amoCAB} operon. The variable regions were indicated by a decrease in the average sequence similarity in the alignments, which were represented graphically (Fig. 3). The gene containing the highest number of the variable regions was \textit{amoB} (Fig. 3c), followed by \textit{amoC} (Fig. 3a). Interestingly, the fewer number of variable regions was observed in \textit{amoA} (Fig. 3b), which is the gene traditionally used for molecular studies in AOB.

The primers \textit{amoC58f} and \textit{amoB1179r} were designed in a previous study (Junier et al. 2008a, b) to amplify the largest segment possible of the operon \textit{amoCAB}, including the three genes and the intergenic regions, in betaproteobacterial AOB. The amplification of \textit{amoCAB} with the primers \textit{amoC58f}/\textit{amoB1179r} produced a band of approximately 3 kb with DNA from the following beta-AOB: \textit{N. europaea} ATCC 19718, \textit{N. eutropha} C-71, \textit{N. briensis} C-128, \textit{N. tenuis} Nv1, and \textit{Nitrosomonas} sp. L13 (supplementary Fig. 1). The size of the \textit{amoCAB} products was variable due to the variability of the intergenic region between \textit{amoC} and \textit{amoA} (Norton et al. 2002). Amplification with the
primers amoC58f/amoB1179r did not produce any visible band with gamma-AOB (N. oceani ATCC 19707) or with the type I and type II MOB M. capsulatus Bath NCIMB 11853 and M. parvus OBBP (supplementary Fig. 1). To confirm the specificity of this amplification, PCR products of amoCAB were used as template for amoA PCR with the primers amoA34f/amoA-2R. A single amplicon of 789 bp was obtained with the beta-AOB products, but no amplification was observed in both gamma-AOB and MOB (supplementary Fig. 1).

Amplification of amoCAB with the primers amoC58f/amoB1179r was also tested with environmental samples using DNA from N. europaea as positive control. A PCR product was visible in the amplification with genomic DNA from N. europaea but not in the environmental samples (data not shown). In this case, the primer combination amoA121fgc–amoA359rC was selected for nested amplification because it produced a fragment that could be directly analyzed by DGGE. A fragment of the expected size (around 230 bp) was obtained from all environmental samples, including the positive control (data not shown).

The AOB community composition in the environmental samples was analyzed by DGGE (Fig. 4a) using N. europaea, N. tenuis, and N. briensis as references. Although more than one band was present on the DGGE gel, each culture produced a dominant band at different positions in the gradient: The band from N. europaea was located at around 45%, the band from N. tenuis at 55%, and the one from N. briensis at around 65% of denaturant concentration.

Each environmental sample was characterized by a specific pattern of bands. The most complex patterns were observed in rhizospheric soil and a water sample from Lake Plußsee, whereas in both Lake Schöhssee and the Baltic Sea, just a single band was observed. The most prominent bands from each pattern were excised from the gel and sequenced. All the sequences matched amoA when subjected to BLAST, with the first hit corresponding to an identified species within the Nitrosospira lineage. The sequences were integrated into ARB and compared to all sequences of the region amplified available in GenBank (Fig. 4b). All sequences but one grouped with sequences from Nitrososira species. The sequence from Schöhssee-10 clustered with sequences from Nitrosomonas species. Sequences from soil were closely related to each other. The soil band 2 and the sequence from the Baltic Sea were related to a sequence from uncultured AOB obtained from rhizospheric soil from a pea plantation. Three out of five sequences from Lake Plußsee formed a cluster related to a clone sequence.
from grassland at the Sourhope research station in Scotland that had received partial additions of N fertilizers (Webster et al. 2002). Sequences from the Jordan River were very diverse and did not form a cluster. One of the sequences (Jordan-9) grouped together with a clone from agricultural soil (Avrahami et al. 2003). The band Jordan-8 was distantly related to the cluster of sequences from Lake Plußsee and *Nitrosospira* sp. NpAV. Finally, the band
Jordan-7 grouped with clones from agricultural soil (Avrahami et al. 2003) and N. multiformis.

Amplification and characterization of additional fragments of the amoCAB operon

Because no bands were visible in the initial PCR with the primers amoC58f/amoB1179r in all environmental samples tested, additional primer combinations (Table 1) were used to amplify different regions of the amoCAB operon for their analysis. These combinations were tested with genomic DNA from N. europaea ATCC 19718 and total DNA extracted from a sample taken at the water–sediment interface at Boknis Eck out of the Kiel Fjord in the Baltic Sea. This sample was selected because a previous characterization using the 16S rRNA gene (Kim et al. 2006) and amoA (Kim et al. 2008) showed a diverse community of AOB composed by both Nitrosomonas and Nitrosospira-like sequences. A total of 16 nested amplifications were applied, but only ten of them produced a band of the expected size in both N. europaea and the environmental sample (primer combinations 1, 4, 5, 6, 7, 8, 9, 10, 13, and 14; Fig. 5). With the primer combinations 2, 3, 11, and 12, a product with the expected size was obtained from N. europaea, but no-product or a product with an unexpected size was obtained from the environmental sample (data not shown). Although a band with the expected size was observed in PCR with the primer combinations 15 and 16, the amplification was very inefficient in both N. europaea and the environmental sample (data not shown).

To better characterize the amoCAB products obtained from the environmental sample and to validate the methodological approach, clone libraries were prepared with the following PCR products: amoC58f/amoA349r, 305F/amoA-2R, amoA34f/amoBMr, amoA34f–amoB1179r, amoA-1F/amoB1179r, and amoA802f/amoBMr (primer combinations 1, 5, 7, 8, 10, and 13 in Fig. 5). These combinations were selected because they represent different regions within the amoCAB operon, including amoC (primer combination 1), the intergenic region (IR) between amoC and amoA (primer combinations 1 and 5), amoA (primer combinations 1, 5, 7, 8, and 10), and amoB (primer combinations 7, 8, 10, and 13). Finally, 47 clones were obtained, containing an insert with the expected size, and in all the cases, the cloned products

Fig. 5 Structure of the amoCAB operon in beta-AOB, including the intergenic space (IS) between amoC and amoA and indicating the position of the primers and different nested PCR tested (expected length of fragments is given in parentheses)
corresponded to the different genes of the amoCAB operon (see below).

In the phylogenetic trees, all amoC clones (766 bp) formed a single cluster (cluster C-I) related to Nitrosomonas (Fig. 6a). The corresponding amoA sequences from the same clones also grouped into a single cluster (cluster A-I, Fig. 6b), which contained sequences from products obtained with the primer combinations 5 (one clone), 7 (three clones), 8 (two clones), and 10 (five clones) in addition. The cluster A-I was related to other sequences from Nitrosomonas species, but formed an independent branch from sequences of cultured AOB.

Since many of the amoA sequences obtained here include the starting codon of the gene, the use of an alternative starting codon was analyzed in the clones. It has been noted before that the unusual start codon GTG is preferentially found in Nitrosomonas amoA, in contrast to the standard ATG start found in Nitrosospira strains (Norton et al. 2002). The analysis of the sequences included in the cluster A-I showed the triplet GTG as start codon (data not shown), further supporting their placement in the Nitrosomonas lineage.

Two additional amoA clusters related to Nitrosospira were observed (Fig. 6b). These clusters include most of the clones from the primer combination 5 (nine clones in cluster A-II and two clones in cluster A-III) and some of the clones of the primer combination 7 (two clones in cluster A-III). All these clones have the standard ATG start codon that has been observed in Nitrosospira strains (Norton et al. 2002).

The phylogenetic assignment of amoB sequences was in agreement with those of amoA. The clones in the cluster B-I of amoB (Fig. 6c) phylogenetically corresponded to those in the amoA cluster A-I, whereas the clones in cluster B-II corresponded to those included in amoA cluster A-III. The cluster B-I was related to the Nitrosomonas lineage and included clones from the primer combinations 7 (three clones), 8 (two clones), 10 (five clones), and 13 (five clones). The cluster B-II belonged to the Nitrosospira lineage and contained sequences from primer combinations 7 (two clones) and 13 (five clones).

Non-coding intergenic regions between amoC–amoA and amoA–amoB

In all the clones, including sequences from amoC and amoA, an IR between the two genes was observed that varied from 136 to 428 bp (data not shown).

In all the clones related to Nitrosospira, the amoA was followed immediately by amoB. In contrast, a 1-bp overlap and shift in the open reading frame between amoA and amoB was observed in the clones related to Nitrosomonas. This is in agreement with previous observations of the physical structure of the amoCAB operon, which indicate that in beta-AOB, amoA is either followed immediately by amoB or there is a 1-bp overlap between both genes (Norton et al. 2002). This is in contrast to the situation with gamma-AOB where the amoA and amoB genes are separated by an intergenic region of approximately 65 bp (Alzerreca et al. 1999; Norton et al. 2002).

Discussion

Traditionally, a fragment of 453 bp from amoA has been used to infer the phylogeny (Purkhold et al. 2000, 2003) and to study the diversity of AOB (Rotthauwe et al. 1997). However, this fraction of amoA provides less resolution than the 16S rRNA gene because it is relatively short and highly conserved (Koops et al. 2003; Purkhold et al. 2003). Therefore, one of the main challenges for studying AOB in environmental samples is the search for alternative molecular markers with higher variability, but can still be amplified by PCR. In the present study, the three genes forming the amoCAB operon are proposed as an alternative to study the phylogeny and diversity of betaproteobacterial AOB in environmental samples.

The general topology of the phylogenetic trees obtained with the full-length amoCAB genes agreed with the existing trees for the 16S rRNA gene and the short fragment of amoA (Purkhold et al. 2000, 2003; Aakra et al. 2001a, b). Additionally, specific lineages within the betaproteobacterial AOB that have been defined based on the short deduced AmoA peptide fragment and 16S rRNA gene sequences (Purkhold et al. 2000, 2003) were also observed. In the phylogeny based on the deduced amino acid sequence of the complete AmoA, the lineages N. oligotropha or N. europaea/N. mobilis were identified (Fig. 1a), but the separation of specific lineages within Nitrosospira spp. could not be evaluated because all available sequences correspond to strains from the Nitrosospira cluster 3. Similarly, because there are fewer amoC and amoB sequences available, only the lineages N. europaea/N. mobilis and the Nitrosospira cluster 3 were represented in the phylogenetic trees of AmoC and AmoB, and therefore, a comprehensive comparison with previously reported AmoA and 16S rRNA gene lineages was not possible.

The position of Nitrosospira cryotolerans in the phylogenetic tree changed when the whole AmoA sequence was considered. With the complete AmoA sequences, N. cryotolerans was related to Nitrosomonas sp. JL21 and Nitrosomonas sp. AL212, while it stands alone in the phylogenetic tree with the short AmoA sequences (Purkhold et al. 2003).

Interestingly, two single amoC copies found in the complete genomes of N. europaea ATCC 19718 (Chain et
Fig. 6 Phylogenetic tree based on AmoC (a), AmoA (b), and AmoB (c) sequences obtained from the Baltic Sea water–sediment interface sample. Phylogenies were constructed with the PHYLIP subroutine by Fitch-Margoliash in ARB using sequences from *N. ocean* ATCC 19707 and PmoCAB from *M. capsulatus* str. Bath and *Methylcoccus* sp. SC2 as out groups. Bootstraps values are indicated.
al. 2003) and N. multiformis ATCC 25196 formed an independent cluster separated from the other AmoC homologs in these species. The separation of these two amoC copies might indicate a different origin and evolutionary background or a less constrictive selective pressure for the evolution of these copies that do not belong to the amoCAB operon. Surprisingly, the second copy in N. multiformis ATCC 25196 clustered with other amoC of the same species.

The analysis of the co-evolution of the different genes in the amoCAB operon showed a significant correlation in the evolutionary rates of the individual genes. Although there are evidences of co-evolution between the genes, this does not imply that the evolutionary rates of each individual gene were equal. In fact, evolutionary rates reflected by the slope of the linear regression were highest in amoB, followed by amoA and amoC (Fig. 2b, d, and f). The differences in the evolutionary rates between the amoCAB genes points toward differences that might correlate to the function of the subunits coded by these genes. In the homologous particulate methane monoxygenase (pMMO), it has been established that the subunit coded by pmoB constitutes the soluble component of the otherwise membrane-bound enzyme complex (Lieberman and Rosenzweig 2005; Balasubramanian and Rosenzweig 2007). By analogy, it can be assumed that amoB also codes for a soluble subunit of AMO. If that is the case, amoB might be subject to a different selection pressure compared to amoC and amoA, which code for the subunits involved in the anchoring of the enzyme in the membrane and in formation of the active site.

An additional element that was considered for evaluating whether a gene is a good candidate for a molecular marker is the presence of conserved regions (suitable for primer design) as well as variable regions (e.g., suitable for phylogenetic inference). This study (Fig. 3) shows that amoB contains a considerably higher amount of variable regions, compared to the shorter amoC and amoA. It also shows that the partial amoA region traditionally amplified in community studies is highly conserved, reaffirming the limitations previously signaled in other studies (Koops et al. 2003; Purkhold et al. 2003). Amplifying the whole amoA allows the inclusion of four additional variable regions, increasing the informative positions that can be used for phylogenetic inference.

The results of the sequence analysis indicate that all the genes amoCAB can be equally used as molecular markers for studying AOB. In case all the amoCAB genes are included as molecular markers for AOB in environmental samples, amoC and amoA (being the most conserved) could be recommended for studying less closely related species, whereas amoB, due to its lesser sequence conservation, could be a better molecular marker to resolve closely related species.

Conventional PCR often is not sensitive enough to amplify amoCAB genes from natural habitats with low abundances of AOB (Beman and Francis 2006; Leininger et al. 2006). Therefore, we have selected a nested PCR approach that could, through successive amplification steps, increase the detection limit of amoCAB genes as has been shown previously for the 16S rRNA gene (Ward et al. 1997, 2000; Hastings et al. 1998; O’Mullan and Ward 2005; Kim et al. 2006). Although amoCAB products could be obtained after initial amplification in all beta-AOB strains tested, in environmental samples, nested PCR was always required to obtain PCR products.

The two methodologies tested to determine the identity of these environmental amoCAB fragments (DGGE and cloning and sequencing) confirmed the obtainment of environmental amoCAB sequences. In this case, the sequences analyzed were not intended to fully characterize the community composition, but rather to confirm the specificity of the products obtained. In all cases, the environmental amoCAB sequences obtained were different enough to be clustered separately of cultured strains of beta-AOB, but clearly they belonged to the beta-AOB.

An additional advantage of the approach proposed here is that it allows the amplification not only of the coding regions but also of the non-coding parts of the amoCAB operon. Sequence analyses of cultured AOB species indicate the existence of an IR between amoC and amoA (Norton et al. 2002). In the present study, both IR regions between amoC and amoA, as well as between amoA and amoB, could be observed in some of the clones sequenced and were informative for the placing of the clones in different phylogenetic groups. Although the intergenic region between the 16S and the 23S rRNA genes has been used to study the phylogeny of AOB (Aakra et al. 2001a, b), the phylogenetic relevance of the IR region between amoC and amoA has never been considered for this purpose.

In summary, the approach presented here opens new possibilities to study the diversity and evolution of AOB in the environment, for the following reasons: (1) It enables an increase in the sensitivity of detection by nested PCR targeting the different AMO genes; (2) it allows the amplification of the almost complete amoA gene, with about 300 bp more sequence information than the previous approaches; (3) as amoB turns out to have the highest sequence variability of the three amo genes, this might be a better molecular marker than the most frequently used amoA to resolve closely related species; (4) the coupled study of all three amo genes and the intergenic spacer regions that are under different selection pressure allows a more detailed analysis of the evolutionary processes, which are responsible for the differentiation of AOB communities at different habitats.
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