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Polygenic analysis of ammonia-oxidizing bacteria for completely autotrophic nitrogen removal

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This study investigated the functional microbial community in a sequencing batch biofilm reactor (SBBR) for completely autotrophic nitrogen removal over nitrite (CANON), and analyzed the phylogenetic relationships of the key bacteria, the aerobic ammonia oxidizing bacteria (AOB), with three different markers genes: 16S rDNA, *amoA* and *hao*. The resulting tree topologies from all the markers were inclined to consistence with minor discrepancy. 16S rDNA- and *hao*-based phylogenetic analyses revealed that AOB for completely autotrophic nitrogen removal were related most closely to *Nitrosomonas* sp., and *amoA*-based phylogenetic analysis demonstrated AOB had highest similarity with a group of uncultured bacteria, while 88 to 94% similarity with *Nitrosomonas* sp. was also achieved. Generally, the detection of three different markers revealed that AOB were closely related to the bacteria of *Proteobacteria Nitrosomonas*. This study shows that each of the functional markers (16S rDNA, *amoA* and *hao*) could be used to trace AOB for completely autotrophic nitrogen removal and it is more accurate if more than two markers are used at a time.

Key words: 16S rDNA; *amoA*, completely autotrophic nitrogen removal; *hao*; phylogenetic analysis.

INTRODUCTION

Biological nitrogen removal is generally used for elimination of nitrogen from wastewater since these components can be toxic to aquatic life, and cause oxygen depletion and eutrophication in receiving water (Brauer and Eitzer, 1997). Ammonia is especially abundant in many wastewater streams and its removal is often achieved using nitrification/denitrification systems. Most nitrification/denitrification systems consist of two steps: aerobic nitrification and anaerobic denitrification, and require two separate reactors (Ahn, 2006). Furthermore, heterotrophic denitrification, widely used in wastewater treatment processes, requires an external organic carbon source to provide electron donors when treating low C/N ratio wastewater, or high internal recycle ratios between aerobic reactors when treating high C/N ratio wastewater (Aoi et al., 2005). Although the recently developed anaerobic

ammonia oxidation (Anammox) reaction has the potential to achieve high ammonia-removal rates at low cost, it requires specific operating conditions and adjustment of the ratio between $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ before the Anammox reaction occurs (Aoi et al., 2005).

Recently, completely autotrophic nitrogen removal system, in which simultaneous nitrification and denitrification under aerobic conditions in a single reactor is achieved, has been described and investigated in the laboratories (Gong et al., 2008; Helmer et al., 2001; Olav Sliekers et al., 2003; Strous, 2000; Third et al., 2005). This is performed in a single reactor at oxygen limited conditions, without the production of N_2O or NO and with production of N_2 . In such a system, autotrophic nitrogen removal process was achieved by the close cooperation between

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aerobic ammonia-oxidizing bacteria (AOB) and Anammox bacteria. AOB oxidize ammonia to nitrite, consuming oxygen and creating anoxic conditions that the Anammox bacteria need (Olav Sliekers et al., 2002). The oxidation of ammonia to nitrite by AOB in wastewater treatment plants (WWTPs) is often considered the most sensitive step in the nitrification process (Purkhold et al., 2000). The oxidation of NH_3 is a two-step process catalyzed by ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO), the key enzymes from AOB (Ari et al., 2002). AMO catalyzes the oxidation of NH_3 to NH_2OH and HAO catalyzes the oxidation of NH_2OH to NO_2^- .

AOB are obligate chemolithotrophs that derive all of the reductant required for energy and biosynthesis from the oxidation of NH_3 to nitrite (NO_2^-). In the NH_4^+ -rich environment, AOB play an important role by initiating the conversion of NH_3 to N_2 . AOB also have potential applications in the bioremediation of polluted soils and waters through the indiscriminate action of the monooxygenase that initiates nitrification (Painter, 1986). In view of the significance of AOB in nitrogen removal process, they have been investigated by the molecular technique (Aoi et al., 2005; Gong et al., 2008; Rowan et al., 2003). The 16S rDNA sequences are suitable for providing a comprehensive long-term evolutionary view of prokaryotic taxonomy because of their conservative characters (Rossello-Mora and Amann, 2001), and have proven useful in the discrimination among organisms (Beja et al., 2002; Hollibaugh et al., 2002; Stephen et al., 1996; Utaker et al., 1995; Weisburg et al., 1991). However, the outcome is confusing when examining a single genus by using 16S rDNA (Rotthauwe et al., 1997). For this reason, genes encoding protein, such as *amoA* which codes for the active site of AMO, have been added to the collection of comparative tools used by taxonomists and molecular ecologists for diversity studies (Aoi et al., 2005; Calvo et al., 2005; Gong et al., 2008; Purkhold et al., 2003), and *amoA* has been extensively used for the detection and study of ammonia oxidizers, particularly in natural environments (Aakra et al., 2001a; b; Horz et al., 2000; Juretschko et al., 1998; Rotthauwe et al., 1997). In addition, another protein encoding gene, *hao*, has been considered as a new molecular biological marker for AOB (Shinozaki and Fukui, 2002), because methane-oxidizing bacteria have the particulate methane monooxygenase (*pmo*) gene which resembles the *amo* gene (Holmes et al., 1995).

AOB plays a significant role for completely autotrophic nitrogen removal (Aoi et al., 2005; Gong et al., 2008; Guo et al., 2008), however they are very sensitive to the natural environment (pH, temperature etc.) and are hard to isolate (Hermansson and Lindgren, 2001). In our previous study, the sequencing batch biofilm reactor (SBBR) for completely autotrophic nitrogen removal over nitrite (CANON) was developed and the complete conversion of ammonia into N_2 in a single reactor was achieved (Fang et al., 2007; Guo et al., 2009), and the mechanism responsible for completely autotrophic nitrogen removal was

studied primarily (Yang et al., 2009), but still knowledge about the community of AOB for SBBR completely autotrophic nitrogen removal is very limited. Of the two groups of important functional bacteria for completely autotrophic nitrogen removal, the dominant Anammox bacteria community has already been identified (Huang et al., 2010). The purpose of this study, therefore, was to investigate the community of AOB for completely autotrophic nitrogen removal and to analyze the phylogenetic relationships. The phylogenetic tree topologies based on 16S rDNA, *amoA* and *hao* from AOB for the process were used for phylogenetic relationship analysis, respectively. The phylogenetic trees constructed from each gene were then weighted against the composite sequence dataset to identify the marker that best reproduced the information resulting from the polygenic tree.

MATERIALS AND METHODS

Total DNA extraction from the sludge

Details of the SBBR for completely autotrophic nitrogen removal system operation have been described (Guo et al., 2009). The seed sludge samples used for total DNA extraction were obtained from the bottom of the reactor, which had been in a stable operation with 90% ammonia conversion ration and 80% total nitrogen removal ration. The total genomic DNA extraction was performed as previously described (Huang et al., 2009) with minor modification. To isolate purified total genomic DNA from the enriched sludge, polyvinylpyrrolidone was used to remove the humic acid contents. The total genomic DNA was obtained by lysing microbes in the soil sample through a series of lysozyme, sodium dodecyl sulfate, and rapid freeze-thaw treatments.

PCR amplification of 16S rDNA, *amoA* and *hao*

Primers aobF/aobR, *amoA*-F/*amoA*-R, and *hao*-F/*hao*-R for 16S rDNA, *amoA* and *hao*, respectively were designed according to the reported nine ammonia oxidizing strains within the β subdivision of the *Proteobacteria* (Head et al., 1993; Woese et al., 1984), using the Primer Express 1.0 (PE Applied Biosystems, Foster City, CA, USA). For 16S rDNA, aobF of 5'-CGAAAGATGTGCTAATACCG-3' and aobR of 5'-TGTGAAGCCCTACCCATAA-3' were used. The amplification of 16S rDNA partial sequence of AOB was performed as follows. The reaction volumes (25 μl) consisted of 1 $\times$ PCR buffer, 0.2 mmol l^{-1} dNTPs, 0.2 $\mu\text{mol l}^{-1}$ of each of the primers, 0.5 U of *pfu* polymerase (Promega Corp., Wisconsin, USA) and 1 μl of the total DNA from the sludge as the templates. The amplification was conducted with an initial denaturing step at 94°C for 4 min, followed by 30 cycles (94°C for 30 s, 62°C for 30 s, 72°C for 2 min) and 72°C for 5 min and a final elongation step at 72°C.

DNA sequence of *amoA* was amplified using the following primers: *amoA*-F (5'-GTGAGTATATTTAGAACGGAAGA-3') and *amoA*-R (5'-TTTATTTGATCCCCCTGG-3'). Reactions were performed as described above. The standard thermal profile used for the amplification of the *amoA* target sequence was as follows: 3 min at 94°C, and then 30 cycles consisting of 30 s at 94°C, 30 s at 48°C, 2 min at 72°C, and a final cycle 5 min at 72°C.

The full length sequence of the *hao* was amplified by using the following primers: *hao*-F (5'-CGGAGGAGAGAGATGAGAATAG-3') and *hao*-R (5'-CGGGTCGGTTGTCACTGCGGT-3'). PCR reactions were performed in 20 μl containing 1 $\times$ GC buffer (TaKaRa Shuzo, Shiga, Japan), 0.4 mmol l^{-1} dNTPs, 0.1 $\mu\text{mol l}^{-1}$ of each primer, 0.5 U of *Pfu* polymerase (Promega Corp., Wisconsin, USA) and 0.8 μl of template DNA. The initial denaturation at 94°C for 3 min was

Table 1. Sequences for 16S rDNA used in this study.

NCBI GenBank accession number	Organism	Reference
AB079053	<i>Nitrosomonas</i> sp. ENI-11	Yamagata et al., 1999
GQ451713	<i>Nitrosomonas europaea</i> strain ATCC 25978	Woese et al., 1984
AF353160	<i>Nitrosomonas europaea</i>	Takahashi, et al. Unpublished
AB070983	<i>Nitrosomonas europaea</i>	Shinozaki and Fukui, 2002
AF338211	<i>Nitrosomonas</i> sp. WH-2	Voytek, 1996
EU267433	Uncultured bacterium clone IIEA1-rp-20 Nit	Beier, et al. Unpublished
AF210051	Uncultured β proteobacterium HB3	Baribeau et al., 2000
DQ988297	Uncultured bacterium clone LR A2-16	Yan, Unpublished
AF037106	<i>Nitrosomonas europaea</i>	Juretschko et al., 1998
AB430334	Uncultured bacterium	Chuang, Unpublished
AJ224941	<i>Nitrosomonas</i> sp. KOII-21	van der Meer, Unpublished
AY135356	Ammonia-oxidizing bacterium NS500-9	Fouratt et. al., 2003
AF363292	<i>Nitrosomonas</i> sp. DYS317	Takahashi, et al. Unpublished
AF353164	<i>Nitrosomonas</i> sp. DYS323	Takahashi, et al. Unpublished
AB093545	<i>Nitrosomonas</i> sp.	Matsuba, et al. Unpublished
AF386750	<i>Nitrosomonas</i> sp. R5c88	Burrell et. al., 2001
AF386749	<i>Nitrosomonas</i> sp. R5c47	Burrell et. al., 2001
AY958676	<i>Nitrosomonas</i> sp. ML1	Burrell et.al., 2001

followed by 30 cycles consisting of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min and the final extension at 72°C for 10 min. Aliquots (5 μ l) of the PCR products of 16S rDNA, *amoA* and *hao* were electrophoresed and visualized in 1% agarose gels by using standard electrophoresis procedures.

Phylogenetic analyses

The nucleotide sequences for 16S rDNA, *amoA* and *hao* genes have been submitted to GenBank database under accession numbers HQ144199, HM473177 and HQ174563, respectively. For phylogenetic analysis, the homologues of 16S rDNA (Table 1), *amoA* (Table 2) and *hao* (Table 3) genes were retrieved from DDBJ/EMBL/GenBank International Nucleotide Data Banks using 16S rDNA (HQ144199), *amoA* (HM473177) and *hao* (HQ174563) as queries, respectively. The collected data was used to construct the phylogenetic trees. Neighbor-joining (NJ) phylogenetic trees for 16S rDNA, *amoA*, and *hao* genes were generated from the corresponding matrix of nucleotide divergence between sequences using the program MEGA5.0, respectively. Confidence in the branching points was obtained with 100 bootstrap replicates. The GenBank accession numbers for the sequences determined in this study are HQ144199 (sequence for 16S rDNA), HM473177 (sequence for *amoA*) and HQ174563 (sequence for *hao*).

RESULTS

AOB phylogeny inferred from 16S rDNA

A unique DNA fragment of approximately 1100 bp was amplified by primers aobF/aobR from the total DNA extracted from the sludge in the reactor for completely autotrophic nitrogen removal system (Figure 1). DNA sequence analysis of the fragment of 1074 bp revealed

that it was partial sequence of 16S rDNA from AOB. Only sequences of more than 98% in similarity were concerned for phylogenetic analysis (Table 1). The phylogenetic tree based on the similarity of sequences of 16S rDNA was constructed (Figure 2). The phylogenetic inference analysis revealed that AOB for completely autotrophic nitrogen removal formed a monophyletic group with *Nitrosomonas* sp., multiple sequence alignments that showed highest similarities to *Nitrosomonas* sp. (99%). AOB strains having more than 60% DNA-DNA similarity are considered as members of the same species (Purkhold et al., 2003). According to DNA-DNA similarity data, AOB for completely autotrophic nitrogen removal are members of the same species of *Nitrosomonas* sp.

AOB phylogeny inferred from *amoA*

During the past few years, the gene encoding the active site subunit of *amoA* has been exploited increasingly as a marker molecule for AOB diversity research in natural and engineered systems. In this study, the full length of *amoA* was used to characterize the AOB for completely autotrophic nitrogen removal. The primers *amoA*-F/ *amoA*-R produced a fragment of approximately 830 bp (Figure 3). The Phylogenetic trees for *amoA* were calculated from the nucleotide datasets and the *amoA* sequence of AOB from completely autotrophic nitrogen removal system showed the highest similarity (99%) to FJ577880 and FJ577884 (Table 2). The organisms from completely autotrophic nitrogen removal system probably represent a novel lineage in the *amoA*-based topology. In addition, 88 to 94% similarity with the *amoA* gene of *Nitrosomonas*

Table 2. Sequences for *amoA* used in this study.

NCBI GenBank Accession Number	Organism	Reference
AF058692	<i>Nitrosomonas europaea</i>	Homes, 1998)
L08050	<i>Nitrosomonas europaea</i>	McTavish et al., 1993
AF058691	<i>Nitrosomonas europaea</i>	Homes, 1998
AB079055	<i>Nitrosomonas</i> sp. ENI-11	Hirota et al., 2000
AB079054	<i>Nitrosomonas</i> sp. ENI-11	Hirota et al., 2000
AF037107	<i>Nitrosomonas europaea</i>	Juretschko et al., 1998
AF037108	<i>Nitrosococcus mobilis</i>	Juretschko et al., 1998
AJ538124	Uncultured soil bacterium, clone SC-MGR-P2K119	Grimm, Unpublished
AJ539318	Uncultured soil bacterium, clone SC-MGR-R2K04	Grimm, Unpublished
AJ538121	Uncultured soil bacterium, clone SC-MGR-P2K116	Grimm, Unpublished
AJ538111	Uncultured soil bacterium, clone SC-MGR-PK107	Grimm, Unpublished
EU010401	Uncultured bacterium clone JJY-amoA-1FF-001	Bae, Unpublished
U51630	<i>Nitrosomonas eutropha</i>	Norton et al., 2002
AB031869	<i>Nitrosomonas</i> sp. TK794	Yokoyama, 1997
AY177932	<i>Nitrosomonas eutropha</i>	Avrahami et. al., 2003
FJ577884	Uncultured bacterium clone amoA_SBR_JJY_70	Bae, Unpublished
FJ577880	Uncultured bacterium clone amoA_SBR_JJY_61	Bae, Unpublished
AF202649	AmoA anoxic biofilm clone S6	Schmid et al., 2000
GU121142	Uncultured bacterium isolate DGGE gel band M23	Yu, Unpublished
AF532304	Uncultured ammonia-oxidizing bacterium clone RI-5a	Nogueira et al., 2002
AF202654	AmoA anoxic biofilm clone S3	Schmid et al., 2000
FJ577855	Uncultured bacterium clone amoA_SBR_JJY_24	Bae, Unpublished
GQ255557	Uncultured ammonia-oxidizing bacterium clone P44-F5-SP6	Dong, Unpublished

Table 3. Sequences for *hao* used in this study.

NCBI GenBank accession number	Organism	Reference
AB030385	<i>Nitrosomonas</i> sp. ENI-11	(Hirota et al., 2000)
AB030386	<i>Nitrosomonas</i> sp. ENI-11	(Hirota et al., 2000)
AB030387	<i>Nitrosomonas</i> sp. ENI-11	(Hirota et al., 2000)
U04053	<i>Nitrosomonas europaea</i>	(Sayavedra-Soto et al., 1994)
FM163618	Uncultured planctomycete, clone P17	(Schmid et al., 2008)
FJ493766	Uncultured <i>Nitrosomonas</i> sp. clone ISA01354	Chen,C.L., Unpublished
FJ493761	Uncultured <i>Nitrosomonas</i> sp. clone ISA01349	Chen,C.L., Unpublished

sp. was achieved, which demonstrated AOB for completely autotrophic nitrogen removal were also closely related to the bacteria of the β subclass of the *Proteobacteria*, *Nitrosomonas*.

AOB phylogeny inferred from *hao*

With the extending dataset and an increasing number of closely related *amoA* sequences, the limitation of the *amoA* approach as applied now becomes more apparent. Although AOB pure cultures or AOB in environmental samples can be assigned rapidly to some phylogenetic

subgroups within this guild by using the *amoA* approach, the *amoA* fragment analysed does provide less resolution. In this study, therefore, AOB for completely autotrophic nitrogen removal were further analysed by a new marker, the *hao* gene.

The protein coding gene, *hao*, is 1713 bp in length and is expressed as a monocistronic transcript (Sayavedra-Soto et al., 1994). In this study, the full length sequence of *hao*, approximately 1700 bp, was produced by primers hao-F/hao-R (Figure 5). The *hao*-based phylogenetic tree demonstrated that AOB for completely autotrophic nitrogen removal are related most closely to “*Nitrosomonas* sp. ENI-11” (Table 3 and Figure 6). The close relationship

Figure 1. PCR amplification of 16S rDNA partial sequence from uncultured ammonia-oxidizing bacteria for completely autotrophic nitrogen removal. 1, DNA Marker (TIANGEN Biotech, Beijing, China); 2-3, partial sequence for 16S rDNA of ammonia-oxidizing bacterium by PCR; 4, Negative control.

Figure 2. 16S rDNA-based phylogenetic tree of the ammonia-oxidizing bacteria (AOB) for completely autotrophic nitrogen removal and its relatives. The tree includes all isolates for which 16S rDNA gene sequences have higher than 98% of similarities with that of AOB (HQ144199). GenBank accession numbers in the tree represent different organisms (Table 1). Confidence in the branching points was obtained with 100 bootstrap replicates. Most of the nodes are supported with high bootstrap values (>60%).

between AOB for completely autotrophic nitrogen removal and *Nitrosomonas* sp. is also reflected by their high DNA-DNA similarity.

Figure 3. PCR amplification of *amoA* from uncultured ammonia-oxidizing bacteria for completely autotrophic nitrogen removal. 1, DNA Marker (TIANGEN Biotech, Beijing, China); 2-3, *amoA* of ammonia-oxidizing bacterium by PCR; 4, Negative control.

DISCUSSION

The autotrophic ammonia-oxidizing bacteria (AOB) do not represent a monophyletic clade but are members of at least two phylogenetically different groups. The first group is characterized by the members of *Nitrosomonas-Nitrospira* clade in the β subclass of the *Proteobacteria*, and the second group is characterized by the strains of the species *Nitrosococcus oceanus*, and probably, *Nitrosococcus halophilus* in the γ subclass of the *Proteobacteria* (Rotthauwe et al., 1997). The first group exists widely in the environment (Belser, 1979), and in this study, the results show that the “*Nitrosomonas*” clade of the β *proteobacteria* AOB existed in completely autotrophic nitrogen removal system.

The phylogenetic tree is a tool which analyzed the phylogenetic relationship of unknown samples in environment. Distance-based neighbor-joining method was used to construct the phylogenetic tree in this study. Though maximum parsimony trees were found to be more accurate than distance-based neighbor-joining method to analyze the AOB (Calvó et al., 2005), the neighbor-joining method was more widely used (Aakra et al., 2001a; Purkhold et al., 2000; Purkhold et al., 2003; Tabei and Ueno, 2010), and neighbor-joining method is proposed for reconstructing phylogenetic trees from evolutionary distance data (Saitou and Nei, 1987). In this study, the 16S rDNA, *amoA* and *hao*-based phylogenetic trees were constructed. The 16S rDNA and *hao* genes sequences determined showed highest similarities (99%) to sequences of *Nitrosomonas* sp., and the *amoA* gene sequences determined demonstrated highest similarity (99%) to sequences of a new group of “uncultured bacterium”, and was also closely related to *Nitrosomonas* sp. (88 to 94% of similarity).

The phylogenetic relationship of AOB in the environment has been investigated based on the 16S rDNA and *amoA* previously (Purkhold et al., 2000; Purkhold et al., 2003), while this is the first report to characterize AOB for completely autotrophic nitrogen removal with three differ-

Figure 4. *aomA*-based phylogenetic tree of the ammonia-oxidizing bacteria for completely autotrophic nitrogen removal and its relatives. The tree includes all isolates for which *amoA* has higher than 88% of similarity with that of AOB (HM473177). GenBank accession numbers in the tree represent different organisms (Table 2). Confidence in the branching points was obtained with 100 bootstrap replicates. Most of the nodes are supported with high bootstrap values (>50%).

rent markers 16S rDNA, *amoA* and *hao* genes. Taken together, topologies of 16S rDNA and *hao*-based trees were similar, but inconsistent affiliations were also found in the *amoA*-based tree (Figures 2, 4 and 6). The 16S rDNA and *hao*-based phylogenetic analysis revealed that AOB for completely autotrophic nitrogen removal belong to *Nitrosomonas* sp., while the *amoA*-based topology showed the members are related most closely to uncul-

tured bacteria, while higher similarity (88 to 94%) with *Nitrosomonas* sp. was also achieved. Previous results demonstrated a superior resolution of 16S rDNA and *amoA* analysis (Purkhold et al., 2003). Comparison of 16S rDNA, *amoA* and *hao* gene in chemolithotrophic AOB showed that the *hao* gene is more useful than the *amoA* gene as a molecular biological marker for AOB detection from environmental samples (Shinozaki and

Figure 5. PCR amplification of *hao* from uncultured ammonia-oxidizing bacteria for completely autotrophic nitrogen removal. 1, DL5000 DNA Marker (TIANGEN Biotech, Beijing, China); 2-3, *hao* of ammonia-oxidizing bacterium by PCR; 4, Negative control.

Figure 6. *hao*-based phylogenetic tree of the ammonia-oxidizing bacteria for completely autotrophic nitrogen removal and its relatives. The tree includes all isolates for which *hao* has higher than 81% of similarities with that of AOB (HQ174563). GenBank accession numbers in the tree represent different organisms (Table 3). Confidence in the branching points was obtained with 100 bootstrap replicates. Most of the nodes are supported with high bootstrap values (>70%).

Fukui, 2002), because methane-oxidizing bacteria have the particulate methane monooxygenase (*pmo*) gene which resembles the *amo* gene (Holmes et al., 1995) and a sequence similar to the *hao* gene has not been reported in other organisms. Therefore, the *hao* gene may be used in the future if the database of this gene is expanded appropriately. In this study, the *hao* gene was first used as a marker to identify AOB for completely autotrophic nitrogen removal and proved its practicability.

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