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Brief report

Diversity of ammonium-oxidizing bacteria in a granular sludge anaerobic ammonium-oxidizing (anammox) reactor

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Summary

The ammonium-oxidizing microbial community was investigated in a granular sludge anaerobic ammonium-oxidizing (anammox) reactor that was operated for about 1 year with high anaerobic ammonium oxidation activity (up to 0.8 kg NH_4^+-N m^{-3} day^{-1}). A *Planctomycetales*-specific 16S rRNA gene library was constructed to analyse the diversity of the anaerobic ammonium-oxidizing bacteria (AnAOB). Most of the specifically amplified sequences (15/16) were similar to each other (> 99%) but were distantly related to all of the previously recognized sequences (< 94%), with the exception of an unclassified anammox-related clone, KSU-1 (98%). An ammonia monooxygenase (*amoA*) gene library was also analysed to investigate the diversity of 'aerobic' ammonium-oxidizing bacteria (AAOB) from the β-*Proteobacteria*. Most of the *amoA* gene fragments (53/55) clustered in the *Nitrosomonas europaea–Nitrosococcus mobilis* group which has been reported to prevail under oxygen-limiting conditions. The quantitative results from real-time polymerase chain reaction (PCR) amplification showed that the dominant AnAOB comprised approximately 50% of the total bacterial 16S rRNA genes in the reactor, whereas the AAOB of β-*Proteobacteria* represented only about 3%. A large fragment (4008 bp) of the rRNA gene cluster of the dominant AnAOB (AS-1) in this reactor sludge was sequenced and compared with sequences of other *Planctomycetales* including four anammox-related candidate genera. The partial sequence of hydrazine-oxidizing enzyme (*hzo*) of dominant AnAOB was also identified using new designed primers. Based on this analysis, we propose to tentatively name this new AnAOB *Candidatus* 'Jettenia asiatica'.

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Introduction

Ammonia removal under anoxic conditions was discovered in a laboratory-scale denitrification process and was confirmed to be a biologically mediated process (van de Graaf *et al*., 1995). Application of the anaerobic ammonium-oxidizing (anammox) process is especially interesting for the biological nitrogen removal from wastewater containing high nitrogen to carbon ratios. Anammox activity has been documented in many different reactor systems including a rotating biological contactor (RBC) (Helmer *et al*., 2001), a trickling filter (Schmid *et al*., 2000), an airlift completely autotrophic nitrogen removal over nitrite (CANON) reactor (Third *et al*., 2001), a sequential batch reactor (SBR) (Strous *et al*., 1998) and the first full-scale granular sludge anammox process (van der Star *et al*., 2007). Furthermore, the role of anaerobic ammonium-oxidizing bacteria (AnAOB) in the marine nitrogen cycle has gained increased attention because

AnAOB may be responsible for 50% of the loss of fixed nitrogen from the ocean (Kuypers *et al*., 2003).

The AnAOB are autotrophic members of the order *Planctomycetales*, one of the major distinct divisions of *Bacteria*. To date, four candidate genera have been reported to perform anammox reactions, including *Candidatus* 'Brocadia' (Strous *et al*., 1999a; Kuenen and Jetten, 2001), *Candidatus* 'Kuenenia' (Schmid *et al*., 2000; Egli *et al*., 2003), *Candidatus* 'Scalindua' (Kuypers *et al*., 2003) and *Candidatus* 'Anammoxoglobus' (Kartal *et al*., 2007a). All of these genera were identified from wastewater treatment biofilms or sludges except some of *Candidatus* 'Scalindua' were identified from marine oxygen minimum zone and many anoxic marine sediments (Kuypers *et al*., 2003; Schmid *et al*., 2007). AnAOB grow very slowly and many have not yet been isolated; therefore, molecular ecological techniques such as fluorescence *in situ* hybridization (FISH) and specific polymerase chain reaction (PCR) amplification are essential for future research on these bacteria (Schmid *et al*., 2005; Tsushima *et al*., 2007a). The use of these molecular ecological tools is based on the documented rRNA sequences from AnAOB. The identification of *Candidatus* 'Scalindua' and *Candidatus* 'Anammoxoglobus' made it clear that the general anammox probe, AMX820, which has been widely used for the measurement of AnAOB from wastewater treatments (Egli *et al*., 2003; Nielsen *et al*., 2005; Chamchoi and Nitisoravut, 2007), only identifies a subset of AnAOB in nature (Schmid *et al*., 2003; Kartal *et al*., 2007b). Furthermore, recent studies using dedicated PCR amplification have documented the presence of substantial amounts of AnAOB in different man-made and natural ecosystems (Penton *et al*., 2006; Innerebner *et al*., 2007; Tsushima *et al*., 2007b). The identification of new AnAOB would increase the genetic and ecological spectrum of AnAOB known, and would be helpful to facilitate the improvement of monitoring of anammox-related bacteria in wastewater treatment systems.

In addition to these AnAOB, some 'aerobic' ammonium-oxidizing bacteria (AAOB) of the β-*Proteobacteria*, such as *Nitrosomonas eutropha* were also recently found to have anaerobic ammonium-oxidizing activity that uses nitrogen dioxide as the electron acceptor (Schmidt and Bock, 1997; Schmidt *et al*., 2002a). In oxygen-limited reactors such as the CANON process, the AAOB convert part of the ammonium to nitrite, which is then used by AnAOB (Third *et al*., 2001). The distribution and activity of AAOB and AnAOB in CANON aggregates were investigated using microelectrodes. It appeared that AnAOB were mostly present in the larger aggregates with low oxygen penetration, while AAOB were present in the smaller aggregates with high oxygen availability. Furthermore, the presence and diversity of both AnAOB and AAOB in anoxic or oxygen-limited ammonium oxidizing reactors have been documented in RBC-type reactor systems (Egli *et al*., 2003; Gieseke *et al*., 2003; Pynaert *et al*., 2003; Tsushima *et al*., 2007b). Together these studies show that cooperation between AnAOB and AAOB can be important for efficient nitrogen removal in different type of reactor systems.

In this study, we analysed anammox activity in a granular sludge anammox reactor that had been operating about 1 year. We determined the abundance and diversity of AnAOB and AAOB by analysing 16S rRNA and *amoA* clone libraries and quantifying their relative composition using real-time PCR. We found that a new type of AnAOB was dominant in this reactor and propose it as a new candidate AnAOB based on a phylogenetic analysis.

Results and discussion

Characterization of the anammox reactor and biomass

The characteristics of the granular sludge after the periods of acclimation and stable operation were determined using batch culture. In batch experiments, the optimum temperature and pH for anammox activity of the sludge from the reactor were 30–35°C and 8.0–8.5 respectively. These values are quite similar to those previously reported for *Candidatus* 'Kuenenia stuttgartiensis' and *Candidatus* 'Brocadia anammoxidans' (Strous *et al*., 1998; Egli *et al*., 2001). The anammox activity for ammonium oxidation at pH 8.3 and 30°C was 14.6 mmol [g volatile suspended solids (VSS)]$^{-1}$ day^{-1}, and the nitrite oxidation was 21.3 mmol (g VSS)$^{-1}$ day^{-1} (Fig. 1). These values are somewhat lower than those reported for *Candidatus* 'Brocadia anammoxidans' but this may be caused by different community composition (see below). In contrast to previous reports, the anammox activity of the present anammox biomass was not inhibited by high

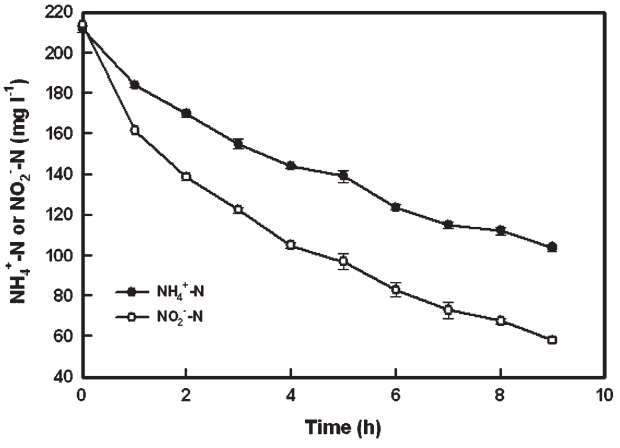

Fig. 1. Batch test of anammox activity in the sludge from the anammox reactor at 377 days.

concentrations (> 7 mM) of nitrite (Strous *et al*., 1999b). The stoichiometric ratio of ammonium to nitrite (1:1.46) was somewhat higher than the reported ratio (1:1.32) in a SBR (Strous *et al*., 1998), but similar to the results (1:1. 45) reported in a RBC (Wyffels *et al*., 2004), indicating that other nitrite-reducing reactions may occur in the system. The concentration of nitrate was below the detection limit, again indicating that there may be surplus electron donor available in the system to drive additional nitrite and nitrate reduction. In agreement with the recent studies suggesting that some specific anammox strains might be able to use organic acids such as formate, acetate or propionate to reduce nitrate or nitrite, it is possible that the AnAOB in the reactor could be responsible for the additional nitrite and nitrate reduction (Kartal *et al*., 2007a,b). Transmission electron microscope (TEM) image of the reactor biomass shows cells with the typical anammox cell structure including anammoxosomes (data not shown). Taken together, these results strongly indicate that the reactor was enriched with AnAOB during our operating conditions.

Diversity of AnAOB

In order to construct a 16S rRNA gene library, the 16S rRNA gene sequences of planctomycetes in the biomass were specifically amplified with the PCR primers PLA-46F and 1390R (Chouari *et al*., 2003). From 16 randomly sequenced clones in the library, 15 sequences were found to be highly similar to each other (more than 99.5% sequence similarity). The presence of one major type of AnAOB has been previously reported in other enrichment cultures and wastewater treatment plants (Schmid *et al*., 2000; Egli *et al*., 2001; Kartal *et al*., 2007a). A 16S rRNA gene (P8) representative was selected from the retrieved anammox sequences for further characterization. Sequence P8 was distantly related to all of the previously recognized sequences (lower than 94% 16S rRNA gene sequence similarity including *Candidatus* 'Anammoxoglobus propionicus'), with the exception of strain KSU-1 (97% 16S rRNA gene sequence similarity). Strain KSU-1 was identified as the dominant bacterial species in a biofilm anammox reactor using non-woven carrier material (Fujii *et al*., 2002). The phylogenetic analysis of sequence P8 (Fig. 2) also showed that the 15 sequences closely branched to known anammox genera and formed a sequence cluster with clone KSU-1. The sequence of one other clone, P4, clustered with *Candidatus* 'Brocadia fulgida', indicating that there could be another subset of AnAOB present in the reactor system in smaller amounts. Zhang and colleagues (2007) also operated up-flow anaerobic reactor for about 25 days using river sediment as inoculum; however, the major AnAOB in that enrichment showed high similarity (95%) to *Candidatus* 'Scalindua'. The difference of AnAOB type may not only be caused by the difference of granulation in the reactor, but also by the difference of the environmental conditions in used river sediment.

Hydrazine-oxidizing enzyme (HZO) is an important protein for the anammox activity, because it is assumed to oxidize the unique anammox intermediate hydrazine to dinitrogen gas. HZO was isolated from anammox enrich-

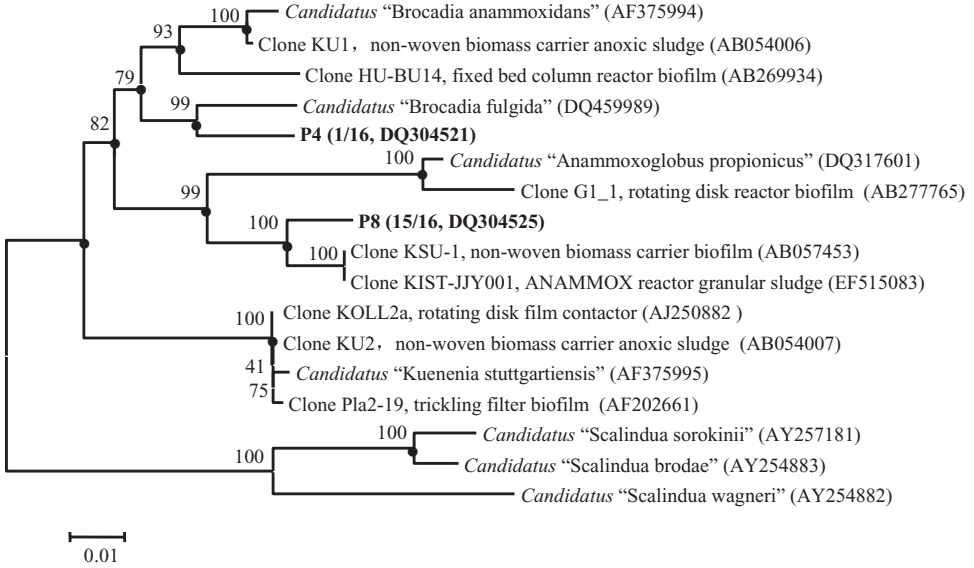

Fig. 2. Phylogenetic neighbour-joining tree reflecting the relationships between the anammox bacteria based on 16S rRNA genes amplified from the anammox reactor sludge using *Planctomycetales*-specific primers. Dots indicate generic branches that were also recovered using maximum-parsimony algorithms. Numbers at the nodes are percentages of bootstrap values based on 1000 re-samplings. Branch lengths correspond to sequence differences as indicated by the scale bar. The GenBank accession numbers are indicated.

ment cultures (Schalk *et al*., 2000; Shimamura *et al*., 2007), and the related sequences were identified in the genome of *Candidatus* 'Kuenenia stuttgartiensis' (Strous *et al*., 2006) and amplified from anammox strain KSU-1 (Shimamura *et al*., 2007). The genes encoding HZO in strain KSU-1 showed high similarity (88–89% on amino acid basis) to two octaheme cytochrome *c* gene products present in the genome assembly of *Candidatus* 'Kuenenia stuttgartiensis' (Shimamura *et al*., 2007). On the basis of the available gene sequences we designed suitable degenerate primers and amplified *hzo* genes from DNA extracted from the anammox granular biomass, and constructed clone library. From 41 randomly sequenced clones in the library, amino acid sequence of clone ANAHZO-f1 with 29 clone sequences showed 98% similarity with the HzoA and HzoB sequences of KSU-1, and 90% similarity with the two octaheme HZO-related ORFs (kustc0694 and kustd1340) from *Candidatus* 'Kuenenia stuttgartiensis'. Other two types of clones, clone ANAHZO-3 with seven clone sequences and clone ANAHZO-4 with two clone sequences, showed 97.5% amino acid similarity with each other, and showed 94% similarity to the HzoA and HzoB sequences of KSU-1 and 93% similarity with clone ANAHZO-f1. This diversity reflected the diversity obtained with the *Planctomycetales*-specific clone library, and suggested that the AS-1 anammox bacteria are the dominant AnAOB in this reactor.

Diversity of AAOB

In addition to AnAOB, AAOB have been reported to be present and possibly to contribute to anaerobic ammonium oxidation (Schmidt *et al*., 2002a). The diversity of AAOB in the reactor biomass was therefore determined using an *amoA* clone library. Fifty-five randomly selected clones from the *amoA* library were sequenced and grouped into five operational taxonomic units (OTU) according to their sequence similarity with *amoA* gene (> 99%). The sequences of *amoA* gene were translated into amino acid sequences (AmoA) for phylogenetic analysis (Fig. 3). About 56% (31/55) of the clones grouped together and are represented by clone A25. The sequence of A25 is very similar to that of the isolate K5F identified from an enriched anammox biofilm, and to ST-B-gene-2 which was isolated under oxygen-limited conditions (Ebie *et al*., 2004). About 18% (10/55) of the clones grouped together and are represented by *amoA* clone A7. The sequence of A7 was similar to that of clone LDD56-8; this group of clones was reported to be the main AAOB in ammonium-oxidizing activated sludge containing a low dissolved oxygen concentration (0.6% oxygen) (Park and Noguera, 2004). Another 18% (10/55) of the clones were very similar to each other (A24) and had a 98% sequence similarity to those of *Nitrosomonas europaea* M103 (Pynaert *et al*., 2003). In that study, it was reported that most (about 98%) of the clones in an AAOB

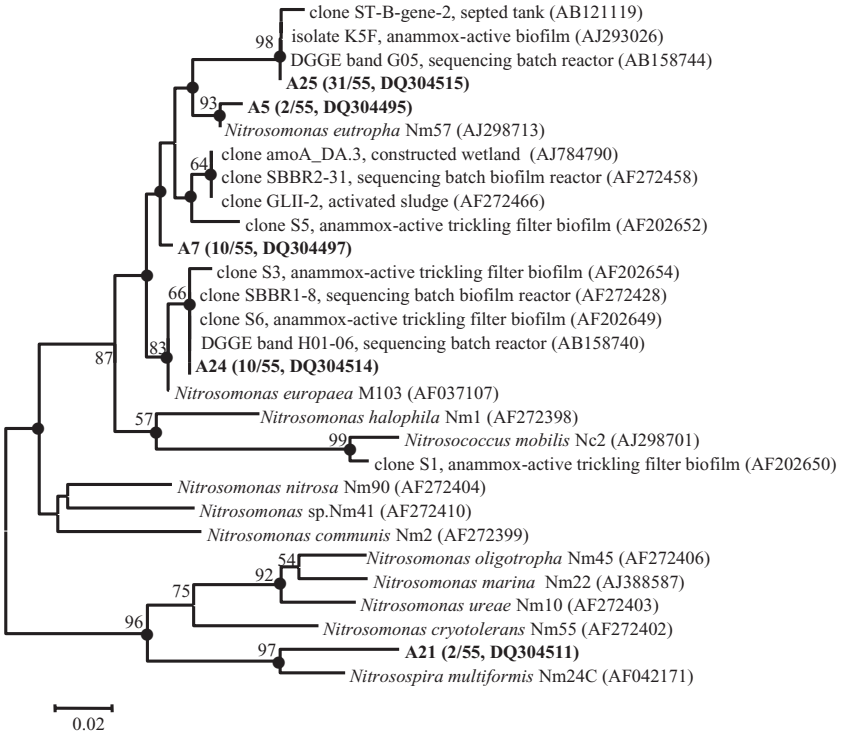

Fig. 3. Phylogenetic neighbour-joining tree based on 128-amino-acid residue sequences of *amoA* retrieved from this study and representative database sequences. Dots indicate generic branches that were also recovered using maximum-parsimony algorithms. Numbers at the nodes are percentages of bootstrap values based on 1000 re-samplings; only values greater than 50% are shown. Branch lengths correspond to sequence differences as indicated by the scale bar. The GenBank accession numbers are indicated.

clone library obtained from the biofilm of an autotrophic ammonium-oxidizing rotating biological contactor clustered with *N. europaea* M103 (Pynaert *et al*., 2003). The two groups that were represented by clones A7 and A24 also clustered together with the AAOB groups of clones S5 and S3 respectively; these clones were reported to be part of the main AAOB in the biofilm of the reactor from which the *Candidatus* 'Kuenenia stuttgartiensis' was identified (Schmid *et al*., 2000). About 4% (2/55) of clones (A5) clustered with *N. eutropha* Nm57. The two remaining clones were most similar to the AmoA of *Nitrosolobus multiformis*. The role of AAOB in anoxic reactor systems is not precisely known. Previously, it was assumed that AAOB were able to survive for long periods of time without oxygen, but more recently there are strong indications that AAOB may also have an anaerobic metabolism that uses nitrite as the terminal electron acceptor (Schmidt *et al*., 2002b). Furthermore, there have been reports that *N. europaea*-related AAOB species were detected in a full-scale biological contactor treating landfill leachate, which showed nitrogen losses, despite a low concentration of dissolved oxygen (Helmer *et al*., 2001; Gieseke *et al*., 2003).

Quantification of AnAOB and AAOB

Fluorescence *in situ* hybridization has been frequently used to quantify AnAOB in various reactor systems (Schmid *et al*., 2005); however, this analysis was often hampered by high levels of autofluorescence and strong clustering of the cells in the granules. A real-time PCR protocol was therefore developed and has been successfully used to quantify both AAOB and AnAOB in the biomass of this anammox reactor (Harms *et al*., 2003; van der Star *et al*., 2007; Tsushima *et al*., 2007a).

One microgram of DNA that was extracted from the reactor sludge yielded about $4.8 \pm 0.2 \times 10^{10}$ copies of the bacterial 16S rRNA genes, $2.4 \pm 0.3 \times 10^{10}$ copies of the P8-type AnAOB 16S rRNA gene and $3.1 \pm 0.4 \times 10^{9}$ copies of the *amoA* gene. Based on these results, the AnAOB-type 16S rRNA gene represented about 51 ± 8% of the total bacterial 16S rRNA genes. The *in situ* analysis of the AnAOB population using the general anammox probe S-*-Amx0368-a-A-18 (Schmid *et al*., 2003) also indicated that AnAOB were the most dominant bacteria in the biomass of the reactor (data not shown). These results correlate well with the somewhat lower observed maximum anammox activity of the biomass (12 nmol min^{-1} mg^{-1}) compared with the 60 nmol min^{-1} mg^{-1} activity of highly purified anammox preparations (Strous *et al*., 1999b).

If *N. europaea/eutropha*-like AAOB are assumed to contain two copies of the *amo* gene cluster (Norton *et al*., 2002) and one copy of 16S rRNA gene per cell (Klappenbach *et al*., 2001), the copy number of the 16S rRNA gene of AAOB would be equal to about $1.6 \pm 0.2 \times 10^{9}$. This indicates that the AAOB 16S rRNA gene copy number would make up not more than about 3% of the total 16S rRNA genes. Thus, the specific anaerobic rate of AAOB would be about 0.13 mmol (g protein)$^{-1}$ day^{-1} (de Bruijn *et al*., 1995), and the contribution of AAOB in the present study is lower than 1% of anammox activity [14.6 mmol (g VSS)$^{-1}$ day^{-1}]. Also the ratio of nitrite to ammonia is in accordance with the expected anammox stoichiometry. Taken together these results suggest that the main ammonium-oxidizing activity in the currently investigated reactor system could be attributed to the AnAOB. Egli and colleagues (2003) reported that in a biofilm obtained from a RBC treating ammonium-rich wastewater without organic carbon, AAOB and AnAOB were present in similar amounts of around 20–30%, but most AAOB were present at the oxic surface of the biofilm. In our anammox reactor, there was no oxygen gradient, explaining why AAOB only formed a minor group in this reactor.

Identification and proposal of a new type of AnAOB

The dominant AnAOB in our biomass (represented by clone P8) was only distantly related (< 94% similarity) to the four known candidate anammox genera. We therefore predicted that the dominant AnAOB would be a new type of AnAOB. To obtain more information, we amplified and cloned a large rRNA gene fragment that included 16S rRNA, ISR (intergenic spacer region) and about 2000 bases of 23S rRNA (Schmid *et al*., 2001). The 16S rRNA gene from clone AS-1 completely matched the sequence of clone P8; therefore, clone AS-1 was then fully sequenced (4008 bp). The arrangement of 16S and 23S rRNA genes in clone AS-1 was the same as that in *Cadidatus* 'Brocadia anammoxidans', *Candidatus* 'Kuenenia stuttgartiensis' and the genus *Isosphaera*. In contrast, the 16S and 23S rRNA genes are separated in three other genera of *Planctomycetales* (*Planctomyces*, *Pirellula* and *Gemmata*) (Schmid *et al*., 2001). Inspection of the 16S rRNA gene sequences of clone AS-1 and KSU-1 revealed that there was an insertion of 16 nucleotides within helix 9 (beginning at *Escherichia coli* position 157) similar to that observed in *Candidatus* 'Anammoxoglobus propionicus', while *Candidatus* 'Brocadia anammoxidans' and *Candidatus* 'Kuenenia stuttgartiensis' had 20-nucleotide insertions. Other *Planctomycetales* bacteria, including *Candidatus* 'Scalindua' spp., have no such insertion in helix 9 (Fig. 4). Figure 4 also shows that primer sequence Ana-F contained five inserted nucleotides is unique for AS-1 and KSU-1 and has more than two mismatches with other microorganisms including four AnAOB genera. The predicted secondary structure of the insertion in clones AS-1 and KSU-1 showed two subhelices, 9a and 9b,

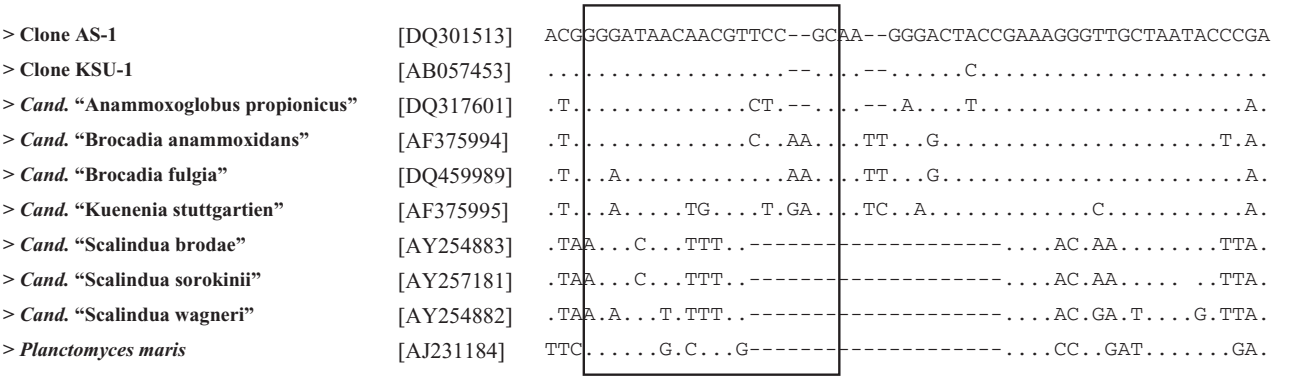

Fig. 4. Multiple 16S rRNA gene sequence alignments of the clone AS-1 with other anammox bacteria and non-anammox *Planctomycetales*. The sequences are related to the secondary structure of helix 9 of the 16S rDNA. Nucleotides that pair with clone AS-1 are indicated by dots and gaps are indicated by dashes. The GenBank accession number of each type of bacteria is represented in square brackets. The window shows the position of specific primer sequence of Ana-F.

which are also present in *Candidatus* 'Brocadia anammoxidans', *Candidatus* 'Kuenenia stuttgartiensis' and *Candidatus* 'Anammoxoglobus propionicus'. Furthermore, an ISR of approximately 450 bp between the 16S and 23S rRNA genes of clone AS-1 contains genes for tRNAs (tAlanine and tIsoleucine); this was also observed in *Candidatus* 'Brocadia anammoxidans' and *Candidatus* 'Kuenenia stuttgartiensis'. The 23S rRNA gene of clone AS-1 was also shown to carry a deletion of helix 58, as found in other AnAOB. Table 1 shows a comparison of 16S rRNA, ISR and 23S rRNA gene sequences from clone AS-1 with the four proposed anammox genera and other planctomycete bacteria for which the sequences are currently available. Clone AS-1 possesses less than 76% gene similarity to the 23S rRNA gene of all of the other planctomycetes except AnAOB, but possesses 90.7% and 88.3% 23S rRNA gene similarity to *Candidatus* 'Brocadia anammoxidans' and *Candidatus* 'Kuenenia stuttgartiensis' respectively.

Based on the results of this study, we propose to provisionally classify the novel AnAOB in the biomass as *Candidatus* 'Jettenia asiatica' according to the taxonomic guidelines (Murray and Schleifer, 1994; Murray and Stackebrandt, 1995). A short description of *Candidatus* 'Jettenia asiatica' (Je.tte'ni.a. N.L. fem. n. Jettenia in honor of Mike S.M. Jetten, for his contribution to the field of anammox; asi.ati'ca L. fem. adj. asiatica, from Asia, where the strain was detected) is as follows: deep-branching within the *Planctomycetales*; not cultivated; basis of assignment, 16S and 23S rRNA sequences (Accession No. DQ301513) and unique sequence of 16S rRNA gene Ana-F (5′-GGG ATA ACA ACG TTC CGC AA-3′); containing genes of hydrazine-oxidizing enzyme (Accession No. EU294365); free-living (anoxic sludge); nitrite tolerant, anaerobic ammonium oxidizer, mesophilic; Quan *et al*., this study.

Conclusions

An anammox reactor that was operated for about 1 year showed a high anaerobic ammonium oxidation activity (0.8 kg NH_4^+-N m^{-3} day^{-1}). The dominant ammonium-oxidizing bacterium was a novel AnAOB comprising about 50% of all of the bacteria in the reactor. This dominant AnAOB was distantly related to all of the currently reported candidate anammox genera, and we propose to name it *Candidatus* 'Jettenia asiatica'. In this reactor, most of AAOB species were clustered in the *N. europaea–Nitrosococcus mobilis* group. AAOB made up about 3% of the bacteria in this reactor.

Experimental procedures

Reactor operation

We used an up-flow anaerobic bioreactor made of polyacrylate with a height of 150 cm and a total working volume of 3.05 l. It was composed of a 1.60 l reaction bed and 1.45 l upper settling bed. River sediment was inoculated into the reactor as the source of seed sludge (the final concentration of VSS was 9.3 g l^{-1}) after selecting granules in the range of 0.6–1.0 mm with a sieve. The reactor was operated at 30–35°C in the continuous flow mode without recycling and was maintained in the dark. The artificial wastewater contained 10 mg l^{-1} KH_2PO_4 and trace elements. The concentration of NH_4^+-N and NO_2^--N was approximately 200 mg l^{-1} each and the loading rate was increased by decreasing the hydraulic retention time. The loading rate was maintained at 0.3 kg of NH_4^+-N m^{-3} day^{-1} until day 130, and was increased from 0.4 to 0.8 kg of NH_4^+-N m^{-3} day^{-1} by day 350. The loading rate was subsequently stably maintained at 0.8 kg of NH_4^+-N m^{-3} day^{-1} with a nitrogen removal rate of more than 80%. The dissolved oxygen in the reactor is lower than the detection limit (0.01 mg l^{-1}). After the periods of acclimation and stable operation, the sludge was sampled to characterize anammox activity and analyse the diversity of the microorganisms involved.

Table 1. Sequence similarities of clone AS-1, four anammox genera and other *Planctomycetales*.

	Anammox bacteria							Other *Planctomycetales*							
	A. propionicus	B. anammoxidans		K. stuttgartiensis			S. brodae	*P. maris*		*P. marina*		*G. obscuriglobus*		*I. pallida*	
rDNA source	16S	16S	ISR	23S	16S	ISR	23S	16S	16S	23S	16S	23S	16S	23S	16S	23S
AS-1	93.6	93.0	75.5	90.7	91.4	67.1	88.2	87.6	76.6	74.6	76.4	71.1	75.4	73.1	72.1	68.8
A. propionicus		91.7	ND[a]	ND	90.3	ND	ND	86.4	76.3	ND	75.6	ND	75.5	ND	74.1	ND
B. anammoxidans					91.4	73.1	89.3	87.7	75.6	74.8	74.2	70.6	76.1	71.6	72.4	68.0
K. stuttgartiensis								88.2	76.6	75.7	76.3	72.5	75.4	73.3	73.4	68.6
S. brodae									76.4	ND	77.3	ND	74.8	ND	72.5	ND

a. ND, not determined because of absent information about the ISR or 23S rRNA gene sequences.
The compared sequences are *Candidatus* 'Anammoxoglobus propionicus' (DQ317601), *Candidatus* 'Brocadia anammoxidans' (AF375994), *Candidatus* 'Kuenenia stuttgartiensis' (AF375995), *Candidatus* 'Scalindua brodae' (AY254883), *Planctomyces maris* (AJ231184, AF245366), *Pirellula marina* (X62912, AF245367), *Gemmata obscuriglobus* (X85248, AF245369), and *Isosphaera pallida* (X64372, AF245370).

Batch test

Batch experiments were performed in 100 ml bottles capped with rubber stoppers. The artificial wastewater described above was used as the medium with 15 mM NH_4^+-N and 15 mM NO_2^--N. The sludge concentration was 1.6 g VSS l^{-1}. The bottles were maintained at a stable temperature in a water bath, shaken at 130 r.p.m., and purged with pure nitrogen gas for 20 min to create anoxic conditions. Stable pH was maintained by titration with hydrochloric acid or sodium hydroxide. During the sampling process, nitrogen gas was flushed through the bottle to prevent air input. After sampling, the nitrogen gas was purged for 15 min. To test the effect of pH, the activities at pH 7.0, 7.5, 7.8, 8.0, 8.3, 8.5 and 9.0 were monitored at 30°C; to test the effect of temperature, the activities at 20°C, 25°C, 30°C, 35°C and 40°C were monitored at pH 8.0. The batch tests were performed in duplicate.

Chemical analyses

The concentration of nitrite and nitrate were determined using a DX-100 ion chromatograph (Dionex, CA) equipped with a conductivity detector. The concentration of ammonium was determined using the colorimetric method. Volatile suspended solids were determined by standard methods, and pH and dissolved oxygen were determined with an electrode.

DNA extraction and PCR amplification

Bulk community DNA was extracted from 1.0 ml of reactor sludge (equal to 10 mg of suspended solids) using liquid nitrogen according to a previously reported protocol (Hurt *et al*., 2001). The *Planctomycetales*-specific 16S rRNA gene was amplified from the extracted DNA using the *Planctomycetales*-specific forward primer PLA-46F and a universal reverse primer, 1390R (Chouari *et al*., 2003). The primer set composed of *amoA*-1F and *amoA*-2R (Rotthauwe *et al*., 1997) was used to amplify the *amoA* gene. The PCR thermal profile was as follows: initial denaturation at 94°C for 2 min; cycles consisting of denaturation at 94°C for 1 min, primer annealing at 59°C for 1 min and extension at 72°C for 1.5 min; and a final elongation step of 10 min (Chouari *et al*., 2003). To decrease the bias during the PCR process, different numbers of cycles (18, 21, 25, 28 and 31 cycles) were used for amplification. Because the products from 18 cycles of PCR also showed smear bands in gel electrophoresis (data not shown), they were used as templates for the construction of clone libraries. A stretch of the rDNA operon including the 16S and 23S rRNA genes and ISR was amplified with PLA-46F and 1037R as previously reported (Schmid *et al*., 2001). The PCR products were purified using an AccuPrep purification kit (Bioneer, Korea).

We designed primer set Ana-hzo1F (5′-TGT GCA TGG TCA ATT GAA AG-3′) and Ana-hzo2R (5′-ACC TCT TC(A/T) GCA GGT GCA T-3′) based on the *hzo* gene sequences of *Candidatus* 'Kuenenia stuttgartiensis' (matching to 826–1859 bp at kustc0694) and the genes amplified from anammox KSU-1-enriched culture (matching to 753–1786 bp at *hzoA* gene). The PCR thermal profile was as follows: initial denaturation at 94°C for 2 min; 30 cycles consisting of dena-

turation at 94°C for 1 min, primer annealing at 53°C for 1 min and extension at 72°C for 1.5 min; and a final elongation step of 10 min.

Cloning and sequencing

The *Planctomycetales*-specific 16S rRNA gene, a large fragment of the rRNA gene operon, the *amoA* gene and the *hzo* gene amplicons were cloned using a TA cloning kit (pGEM-T Easy Vector; Promega) in accordance with the manufacturer's instructions. The clones were grown in Luria–Bertani medium plates supplemented with ampicillin (100 μg ml^{-1}). Clones were randomly selected for further analysis. Cells were lysed by boiling and 1 μl of aliquots were directly used for PCR amplification and sequencing. The sequences of the large fragment of the rRNA gene operon were determined using the forward and reverse primers and internal primers located on the 16S and 23S rRNA gene respectively (Schmid *et al*., 2001). Sequences of 16S rRNA gene were checked for possible chimeras using the CHIMERA_CHECK program at the Ribosomal Database Project website (http://rdp8.cme.msu.edu/html/analyses.html).

Phylogenetic analysis

In order to determine the phylogenetic position of the 16S rRNA and *amoA* gene sequences, they were compared with available database sequences via BLAST search, and the related taxa were obtained from GenBank. The multiple alignments were performed with the CLUSTAL X program (Thompson *et al*., 1997). The resulting alignments were manually checked and corrected when necessary. Gaps in the sequences of 16S rRNA gene were edited and the sequences of amino acid (AmoA) sequences were deduced from the *amoA* gene sequences with the BioEdit program (Hall, 1999). The phylogenetic trees were constructed via the neighbour-joining and maximum-parsimony algorithms with the MEGA 3 program (Kumar *et al*., 2004). The similarity of the mostly complete rRNA gene sequence of clone AS-1 was compared with that of other *Planctomycetales* species, including reported AnAOB. The tRNA gene was found from the large fragment of the rRNA gene operon using tRNAscan-SE software (http://lowelab.cse.ucsc.edu/tRNAscan-SE).

Real-time PCR analysis

To determine the frequency distribution of AnAOB and AAOB in the anammox reactor sludge, real-time PCR amplification was performed using a SYBR Green qPCR Kit (Finnzymes, Finland). Real-time PCR was performed in 96-well optical plates placed in the DNA Engine Opticon System (MJ Research, MA, USA). The primer set composed of *amoA*-1F and *amoA*-2R was used for the amplification of the *amoA* gene. Ana-F (5′-GGG ATA ACA CG TTC CGC AA-3′) and a modified Ana-R (5′-CGA TAC CTA AGT ATC TAG AGT-3′) (Fujii *et al*., 2002), which are specific to the main AnAOB in this study, were used to amplify the 16S rRNA gene of the specific AnAOB. The primer set 1390F (5′-TTG TAC ACA CCG CCC GTC-3′) and 1492R (5′-ACC TTG TTA CGA CTT-3′) was used to quantify the total number of bacterial 16S rRNA gene copies in the biomass from the anammox reactor. The specificity of the PCR amplification was determined by the melting curve and by gel electrophoresis. Cycle thresholds were determined by comparison with standard curves constructed using several concentrations of clone P8 (for the 16S rRNA gene of AnAOB and all bacteria) and clone A25 (for the *amoA* gene of AAOB). Relative copy numbers among target organisms were evaluated. The PCR protocol was as follows: one cycle at 95°C for 15 min and 40 cycles at 94°C for 30 s, 54°C for 30 s and 72°C for 30 s. The R^2 values were greater than 0.98 for all of the curves.

Nucleotide sequence accession numbers

The sequences determined in this study are available in GenBank under Accession No. DQ304491 to DQ304517 (for the clone sequences of *amoA* gene); DQ304518 to DQ304533 (for the clone sequences of *Planctomycetales*-specific 16S rRNA gene), DQ301513 (clone AS-1) and EU294365 to EU294367 (for the clone sequences of AnAOB-specific *hzo* gene).

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