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Community analysis of ammonia-oxidizing bacteria in activated sludge of eight wastewater treatment systems

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Abstract

We investigated the communities of ammonia-oxidizing bacteria (AOB) in activated sludge collected from eight wastewater treatment systems using polymerase chain reaction (PCR) followed by terminal restriction fragment length polymorphism (T-RFLP), cloning, and sequencing of the α-subunit of the ammonia monooxygenase gene (*amoA*). The T-RFLP fingerprint analyses showed that different wastewater treatment systems harbored distinct AOB communities. However, there was no remarkable difference among the AOB T-RFLP profiles from different parts of the same system. The T-RFLP fingerprints showed that a full-scale wastewater treatment plant (WWTP) contained a larger number of dominant AOB species than a pilot-scale reactor. The source of influent affected the AOB community, and the WWTPs treating domestic wastewater contained a higher AOB diversity than those receiving mixed domestic and industrial wastewater. However, the AOB community structure was little affected by the treatment process in this study. Phylogenetic analysis of the cloned *amoA* genes clearly indicated that all the dominant AOB in the systems was closely related to *Nitrosomonas* spp. not to *Nitrosospira* spp. Members of the *Nitrosomonas oligotropha* and *Nitrosomonas communis* clusters were found in all samples, while members of *Nitrosomonas europaea* cluster occurred in some systems.

Key words: activated sludge; ammonia-oxidizing bacteria; T-RFLP; *amoA* gene; wastewater treatment plant

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Introduction

Ammonia can be toxic to aquatic life at relatively low concentrations. It contributes to eutrophication and leads to a significant oxygen demand in receiving waters (Siripong and Rittmann, 2007). An ideal and inexpensive way to remove ammonia is biological nitrification coupled with denitrification, which is widely used in wastewater treatment plants (Geets et al., 2007). Since nitrifiers, especially ammonia-oxidizing bacteria (AOB), are highly sensitive to several environmental and engineering factors, including temperature, pH, dissolved oxygen (DO), and a wide variety of chemical inhibitors (Sofia et al., 2004; Hallin et al., 2005; Satoh et al., 2006), nitrification failure is unfortunately a frequent occurrence in wastewater treatment plants (Siripong and Rittmann, 2007; Qin et al., 2008).

Therefore, a better understanding of the microbial ecology of AOB in wastewater treatment systems could potentially enhance the treatment performance and control (Limpiyakorn et al., 2004; Lim et al., 2008). It would also help engineers to utilize the functional characteristics of the microbial population to model and improve the design and operation of the systems (Gilbride et al., 2006). For instance, early detection of a change in the nitrifier population may indicate the performance inhibition, thereby allowing the operator to take action to prevent washout of these essential bacteria (Horz et al., 2000; Siripong and Rittmann, 2007).

Various approaches, both culture-dependent and -independent, have been applied to analyze and compare the microbial community structure of activated sludge. However, culture-dependent methods are biased by the selection of species which obviously do not represent the real dominance structure (Gilbride et al., 2006; Sanz and Kochling, 2007). Recently, the development of culture-independent molecular techniques, like fluorescence *in situ* hybridization (FISH), denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) have enhanced the ability of researchers to analyze environmental samples. Because of its relatively high resolution and reproducibility (Osborn et al., 2000), T-RFLP has been widely used to assess the AOB community in various engineering

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systems (Regan et al., 2002; Mintie et al., 2003; Park and Noguera, 2004; Bernhard et al., 2005).

A number of recent studies of AOB in wastewater treatment systems suggested that different facilities support different populations and different levels of species richness (Rowan et al., 2003). For example, some researchers have found *Nitrosomonas*-like populations to be the dominant AOB in activated sludge bioreactors treating municipal wastewater (Dionisi et al., 2002; Egli et al., 2003; Park and Noguera, 2004; Hallin et al., 2005; LaPara and Ghosh, 2006), while other researchers have determined that *Nitrosospira*-like populations prevail in some bioreactors (Coskuner and Curtis, 2002; Sofia et al., 2004). The lack of consensus on the specific type of AOB predominant in wastewater treatment plants (WWTPs) suggests that the type of wastewater, as well as characteristics of the WWTP such as aeration intensity, reactor configuration, solids retention time (SRT), and hydraulic retention time (HRT), may establish significantly different environments, and therefore, select for different types of AOB (Park and Noguera, 2004). However, some researchers (Siripong and Rittmann, 2007) have concluded that activated sludge systems providing stable and complete nitrification had significant and similar diversity and functional redundancy among their ammonia-oxidizing bacteria despite differences in temperature, SRT, and other operating conditions.

Recently the geographic distribution of microorganisms has been examined. However, there is still debate as to whether microorganisms exhibit any biogeographic patterns, partly because there are not enough samples from globally distributed sites to settle the debate (Martiny et al., 2006).

To examine whether selection for particular AOB occurs in full-scale wastewater treatment plants, and also to investigate the spatial distribution of AOB and the effects of treatment process, type of wastewater, and plant scale on AOB community structure, we characterized the AOB presented in 8 wastewater treatment systems in Beijing using T-RFLP, cloning, and sequencing of the functional gene *amoA*. This study should also, to some extent, accumulate significant data for addressing the biogeography debate.

1 Materials and methods

1.1 Wastewater treatment systems and sampling

Activated sludge samples were collected from the aeration tanks of eight systems in six wastewater treatment plants, all of which are operated by Beijing Drainage Group Co., Ltd. Among these, system D2 is a pilot-scale system, and the others are all full-scale wastewater tretment plants. Systems A1 and A2 are located in plant A, while systems D1 and D2 are located in plant D. The 8 systems were operated using different treatment processes: A2, B, D2, and E were anaerobic/anoxic/aerobic (A^2O) processes; system A1 was anoxic/anaerobic/aerobic (reverse A^2O) process; system F was an oxidation ditch; and systems C and D were conventional activated sludge (CAS) processes.

Mixed-liquor suspended solids (MLSS) samples were collected from the front, middle and end parts of each aeration tank (except E and F) on March 14, 2007. The wastewater temperatures of the WWTPs were all about 18°C. For systems E and F, we only took 2 samples from the front and end part of each aeration basin. Each sample of 1.5 mL was dispensed into a 2-mL Eppendorf tube and centrifuged at 14,000 ×*g* for 10 min. The supernatant was removed, and the pellet was kept at –20°C until analysis.

1.2 DNA extraction

The pellets of activated sludge samples were washed with sterile high-purify water by centrifuging for 5 min at 15,000 ×*g* three times. Then, DNA extraction was performed using the FastDNA® SPIN Kit for Soil (MP Biotechnology, USA) according to the manufacturer's protocol. We eluted DNA from the kit's column membrane with 100 μL of DNase/Pyrogen-free Water. The genomic DNA concentration was measured with a spectrometer at 260 nm. The purity of genomic DNA was determined by calculating the ratio of the absorbance measured at 260 nm to that measured at 280 nm.

1.3 PCR amplification

The primers *amoA*-1F (5'-GGGGTTTCTACT GGTGGT-3') and *amoA*-2R (5'-CCCCTCKGSAAAGC CTTCTTC-3') (Rotthauwe et al., 1997) were used to amplify a 491 base pairs (bp) fragment of the *amoA* gene of AOB. Each PCR reaction with a final volume of 50 μL contained 5 μL 10× PCR buffer (TaKaRa, Japan), 4 μL deoxynucleotide triphosphate (dNTP) mix (2.5 mmol/L, TaKaRa, Japan), 1.25 U TaKaRa Ex Taq™ enzyme (5 U/μL, TaKaRa, Japan), 1 pmol of each primer and approximately 1–10 ng template DNA. The PCR was performed in a thermocycler (MJ Research, USA) under the following conditions: initial denaturing step of 9 min at 95°C followed by 40 cycles of denaturing (94°C for 2 min), annealing (55°C for 2 min) and elongation (72°C for 2 min). For T-RFLP analysis, PCR amplification of *amoA* was carried out with the forward primer (*amoA*-1F) 5'-labeled with the dye 6-carboxyfluorescein (FAM) (Horz et al., 2000).

1.4 Purification of PCR products and restriction enzyme digestion

The PCR products were verified by electrophoresis on 2% (*W/V*) agarose gels, followed by 15 min of staining with ethidium bromide. Fluorescently labeled PCR products (40 μL) were purified using the QIAquicks PCR purification kit (Qiagen Inc., Canada) and digested with TaqI restriction endonuclease (TaKaRa, Japan) at 65°C for 5 hr followed by 95°C for 20 min. The restriction-digestion mixture contained 8 μL of purified PCR product, 1 μL of enzyme buffer, and 1 μL containing 10 U of restriction endonuclease.

1.5 T-RFLP analysis

The digested DNA was precipitated with 1 μL of 3 mol/L sodium acetate and 20 μL of 95% ethanol, followed

by centrifugation at 14,000 ×*g* for 20 min at 4°C. The DNA pellets were washed with 70% ethanol, and then air dried. The precipitate was mixed with 0.3 μL of GS-500LIZ standard (Applied Biosystems, USA) and 10 μL of deionized formamide (Applied Biosystems, USA). After DNA denaturation at 94°C for 5 min and immediate chilling on ice, the fluorescently labeled terminal restriction fragments (T-RFs) were run through an ABI PRISM 3130-Avant Genetic Analyzer (Applied Biosystems, USA) in the GeneScan mode. The size and intensity of each T-RF (peak height or area) were calculated automatically using GeneMapper software version 3.0 (Applied Biosystems, USA).

In each sample, only peaks over a threshold of 50 fluorescence units were used and T-RFs smaller than 50 bp or larger than 500 bp were excluded from the analysis to avoid the detection of primers and uncertainties of size determination. Because the amount of DNA loaded on the capillary could not be controlled accurately, the sum of all T-RF peak areas in a pattern (total peak area) varied among samples. The relative abundances of T-RFs were standardized in percentage by calculating the ratio of a given peak area to the normalized total peak area of each sample. Peaks with an area less than 1% of the total were reassigned as zero, and the proportion of each remaining peak was recalculated. Peaks with an area less than 2% were removed. The data set was analyzed with a 2% threshold to remove any bias caused by the amount of PCR product (Flores-Mireles et al., 2007).

1.6 Cloning and sequencing

Prior to cloning, the amplified unlabeled 491 bp *amoA* gene fragments were purified using the QIAquick PCR Purification Kit (Qiagen, Germany). Purified PCR products were ligated into the pGEM-T Easy cloning vectors (Promega, USA), and transformed into competent *Escherichia coli* DH5α cells (Tiangen, China) as described in the manufacturer's protocol. Transformants were selected by ampicillin resistance, and blue-white screening was performed to identify clones with inserts. Forty-eight white colonies from each *amoA* gene library were selected and cultivated. Primers T7 and SP6 were used to perform colony PCR and to verify that the insert size was correct. Following PCR confirmation of insert size, the amplified inserts were run on 2% (*W/V*) agarose gels. The samples containing inserts of the estimated size were used for subsequent sequencing. Sequencing was done by a commercial company (Nuosai gene, China).

1.7 Phylogenetic analysis

The software ClustalX 1.81 was used to align sequences of the recovered clones with other published *amoA* sequences and software Mega 4.0 was used to generate a phylogenetic tree using the neighbor joining method.

1.8 Cluster analysis

The relative abundance of each T-RF was considered as a variable. Similarity matrices of AOB as revealed by T-RFLP patterns were calculated with the Squared Euclidean distance coefficient using the software SPSS 13.0.

1.9 Accession numbers for nucleotide sequences

The partial sequences of the *amoA* gene obtained in this study are available from the NCBI database under accession numbers FJ423002-FJ423027.

2 Results

2.1 Mixed-liquor samples and performance of WWTPs

Details of the influent and effluent characteristics and operational parameters of the 8 systems are listed in Table 1. Biological oxygen demand (BOD) in the influents ranged from 175 to 265 mg/L, while ammonium concentrations were between 41.0 and 66 mg/L. The BOD removal efficiencies were more than 92% in all systems, and ammonia removal efficiencies varied from 82% to 93%. Ammonia, nitrite and nitrate concentrations in all the effluents were less than 4, 6, and 13 mg/L, respectively.

2.2 Spatial distribution of AOB communities

We used *amoA*-based T-RFLP to investigate AOB communities present in the 8 wastewater treatment systems. As the T-RFLP profiles showed, different wastewater treat-

Table 1 Summary of overall operating data of 8 wastewater treatment systems*

System	A1	A2	B	C	D1	D2	D3	E	F
Treatment process	Reversed A^2O	A^2O	A^2O	CAS	A^2O	A^2O	AO	CAS	Oxidation ditch
Source of influent	R	R	I + R	R	I + R	I + R	I + R	R	I + R
Influent BOD (mg/L)	254	254	225.5	241	175	181.8	175	265	186.39
Influent NH_4^+-N (mg/L)	41.0	41.0		56.4	45.9	44.5	44.6	66	
Influent TN (mg/L)	52.8	52.8	70.4	57.0	52.5	52.9	52.5	79.3	52.1
Effluent BOD (mg/L)	10.8	10.9	8.9	9.7	11.7	9.9	10.5	8.3	8.8
Effluent NH_4^+-N (mg/L)	1.8	1.9	3	4.0	5.5	5.0	4.7	4.0	4.0
Effluent NO_2^--N (mg/L)	0.3	0.2	0.2	0.2	0.5	0.6	0.1	0.1	0.3
Effluent NO_3^--N (mg/L)	8.7	8.2	9.7	17.8	17.4	13.8	12.7	16.2	4.4
Effluent TN (mg/L)	21.6	20.5	16.5	23	21.45	25.1	22.5	21.5	
MLSS (mg/L)	2821	3330	3120	2284	2433	2428	2793	2241	4630
SRT (day)	13	13	12		11	11	11		16.3
DO (mg/L)	2.61	2.56	2.47	2.20	2.78	2.35	2.36	2.38	2.72

* Each value represents the average of data over 30 days before sampling.
R: residential; I: industrial; BOD: biological oxygen demand; SRT: solid retention time; TN: total nitrogen; DO: dissolved oxygen; MLSS: mixed-liquor suspended solids.

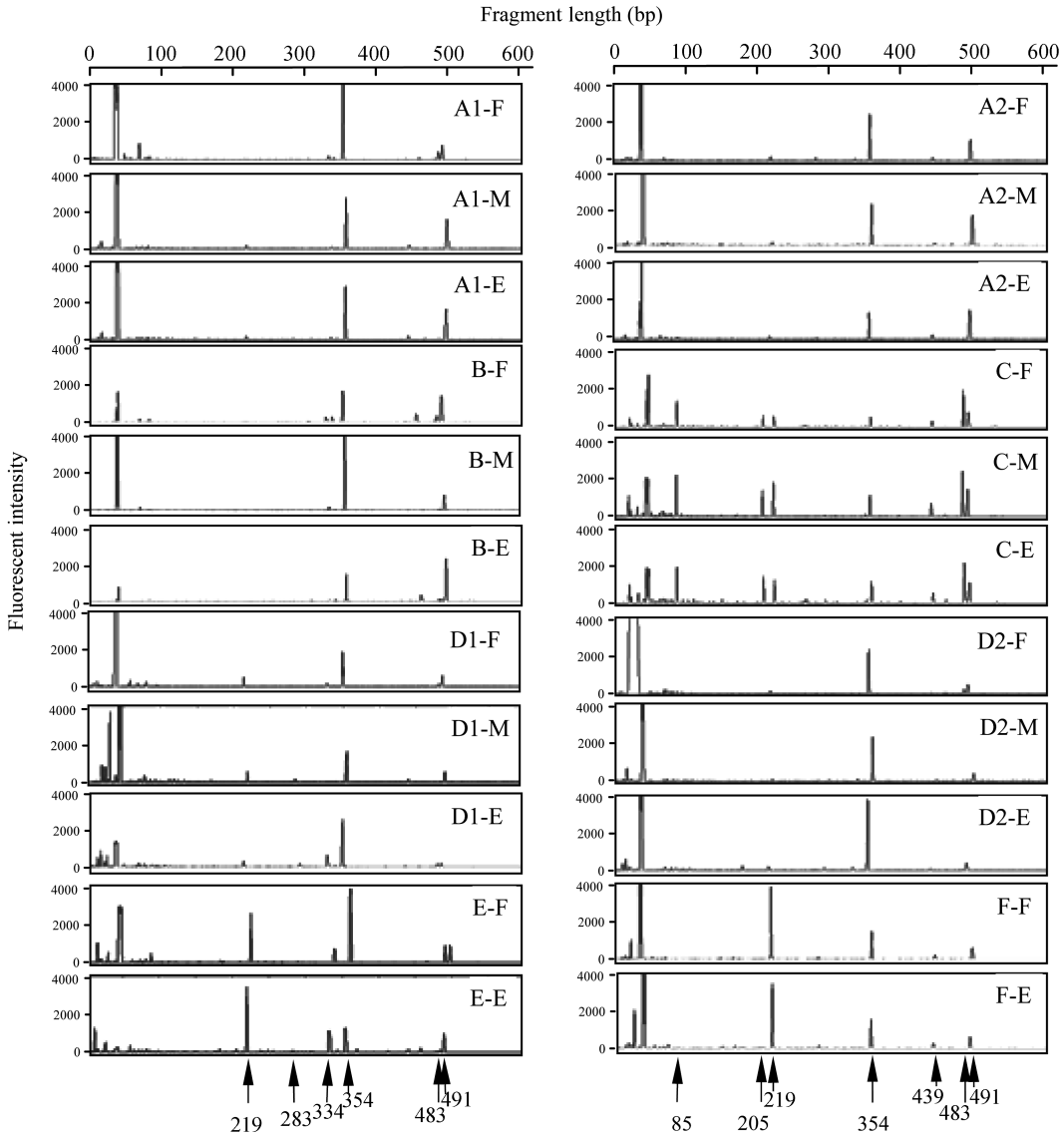

Fig. 1 T-RFLP profiles of *TaqI*-digested *amoA* genes from activated sludge of 8 wastewater treatment systems. The words in the upper right corner of each T-RFLP profile indicate different wastewater systems and sampling locations; the first character and arabic numeral represent the wastewater treatment system, and the character after "-" (F, M, or E) stands for the sampling location (front, middle or end) in the aeration tanks.

ment systems harbored different AOB components (Fig. 1). However, the T-RFLP profiles and the cluster analysis (Figs. 1 and 2) showed that activated sludge samples from different parts of each aeration tank exhibited very high similarity. For example, A2-F, A2-M, and A2-E, which came from different parts of system A, were very similar, all characterized by abundant 354 and 491 bp T-RFs. The AOB community structures of samples taken from different parts of the same aeration tanks in other systems also had high similarity.

2.3 AOB community response to different treatment processes

Plant A has two treatment trains with the A^2/O process (A1) and reverse A^2/O process (A2). A comparison of the T-RFLP profiles between A1 and A2 revealed that the two systems harbored similar AOB populations, all characterized by a large proportion of 354 and 491 bp T-RFs (Fig. 1). Cluster analysis also revealed that the samples from these two systems had a high level of similarity (Fig. 2). This indicates that the AOB community structures of these two systems were not significantly affected by the process. That is, the different processes of the two systems did not appear to select for different communities.

2.4 AOB communities in systems of different scales

Plant D1 and the pilot-scale bioreactor D2 were both located in the same wastewater treatment plant, used the same A^2O process, and received the same wastewater. The predominant peaks in the T-RFLP profiles of D1 were 219, 354 and 491 bp T-RFs, and those of D2 were 354 and 491 bp T-RFs. The profiles from D1 showed a larger number of predominant fragments than those of D2. Although the diversity of dominant T-RFs seemed different, cluster analysis showed that the community composition of AOB in the samples from these two systems was similar (Fig. 2).

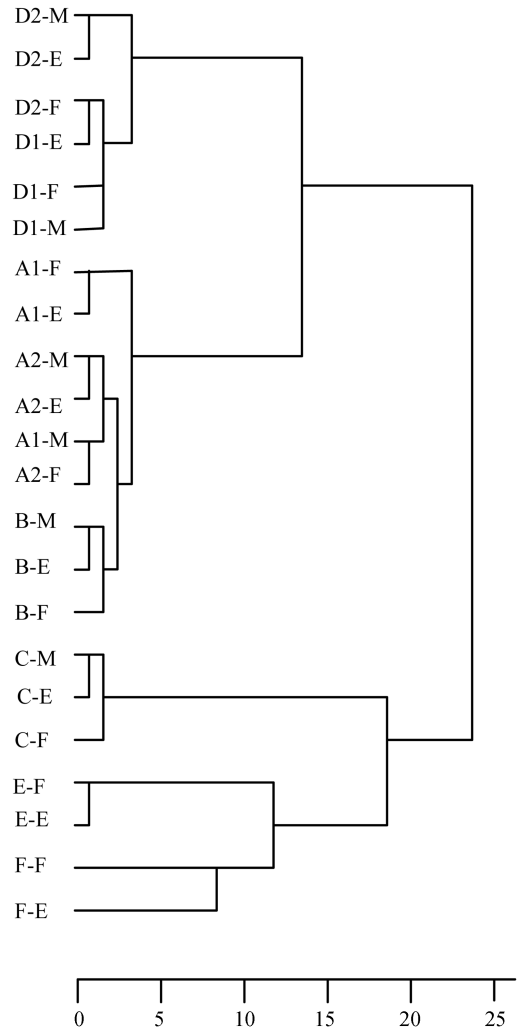

Fig. 2 Hierarchical cluster analysis of T-RFLP profiles.

2.5 AOB communities responding to different sources of influent

Plants A, C and E treated pure domestic wastewater; the other systems treated a mixture of industrial and domestic wastewater. Analysis of the T-RFLP fingerprints from the 8 wastewater systems indicated that the activated sludge samples from domestic wastewater plants (C and E, except A) exhibited a higher AOB diversity than systems treating a mixture of industrial and residential wastewater (Fig. 1). This suggests that the source of influent influences the AOB community.

2.6 Phylogenetic analyses

To identify which species were represented by the T-RFLP peaks, clone libraries were constructed from 6 wastewater treatment systems (A2, B, C, D1, E, F), which were all from different plants.

Forty-eitht white colonies of each *amoA* gene library were selected and cultivated, and then T-RFLP was performed using each *amoA* gene clone as template DNA. Because each clone contained one unique *amoA* gene fragment, each T-RFLP profile had only one peak. The colonies in each sample were classified according to the length of peak, and at least two representative colonies from each category of colonies were sequenced. There are total 126 colonies were sequenced. By combining the results of sequencing the *amoA* gene and the T-RFLP profiles of clones, we could determine the species each peak represented. Our results revealed that in the systems studied the 219, 354, and 491 bp peaks indicated members of *Nitrosomonas europaea* cluster, *Nitrosomonas oligotropha* cluster and *Nitrosomonas communis* cluster respectively. Phylogenetic analysis of the cloned *amoA* genes clearly showed that all the sequences were closely related to *Nitrosomonas* spp., not to *Nitrosospira* spp. (Fig. 3).

3 Discussion

3.1 Spatial distribution of AOB communities

The T-RFLP profiles of AOB (Fig. 1) showed that activated sludge samples from different parts of the same aeration tank exhibited similar T-RFLP patterns. All the wastewater treatment systems studied are activated sludge processes, including A^2O, CAS, and oxidation ditch, which all have a return activated sludge recirculation system. For A^2O, in addition to the return activated sludge recirculation system, an interior recirculation system is also employed for denitrification. The activated sludge recycle systems, combined with the mixing power of the water flow and the powerful aeration in the aeration tanks may accomplish complete mixing of the activated sludge in aeration tanks. Therefore, the different parts of each aeration tank possess similar AOB communities.

3.2 AOB communities responding to different treatment processes

To examine whether full-scale wastewater treatment plants with different treatment processes select for particular AOB, we compared the AOB communities of A1 and A2 systems with different treatment processes (A^2O and reverse A^2O process) receiving identical wastewater. Comparative analysis of the T-RFLP profiles of AOB *amoA* gene from systems A1 and A2 revealed no significant difference between the two reactors. This result seems to contradict the idea that different environments select for different AOB populations. Rowan et al. (2003) found that a trickling filter system had a greater diversity of AOB than a biological aerated filter (BAF), although the two reactors received the same mixed domestic and industrial wastewater. Dytczak et al. (2008) reported that a sequencing batch reactor (SBR) operating under an alternating anoxic/aerobic process harbored a dominance of *Nitrosomonas*, whereas *Nitrosospira* dominated in a strictly aerobic SBR reactor. In our study, despite the different environmental conditions for the AOB in the two processes (A^2O and reversed A^2O process), the total selective pressure in the two trains may not strong enough to induce a population shift.

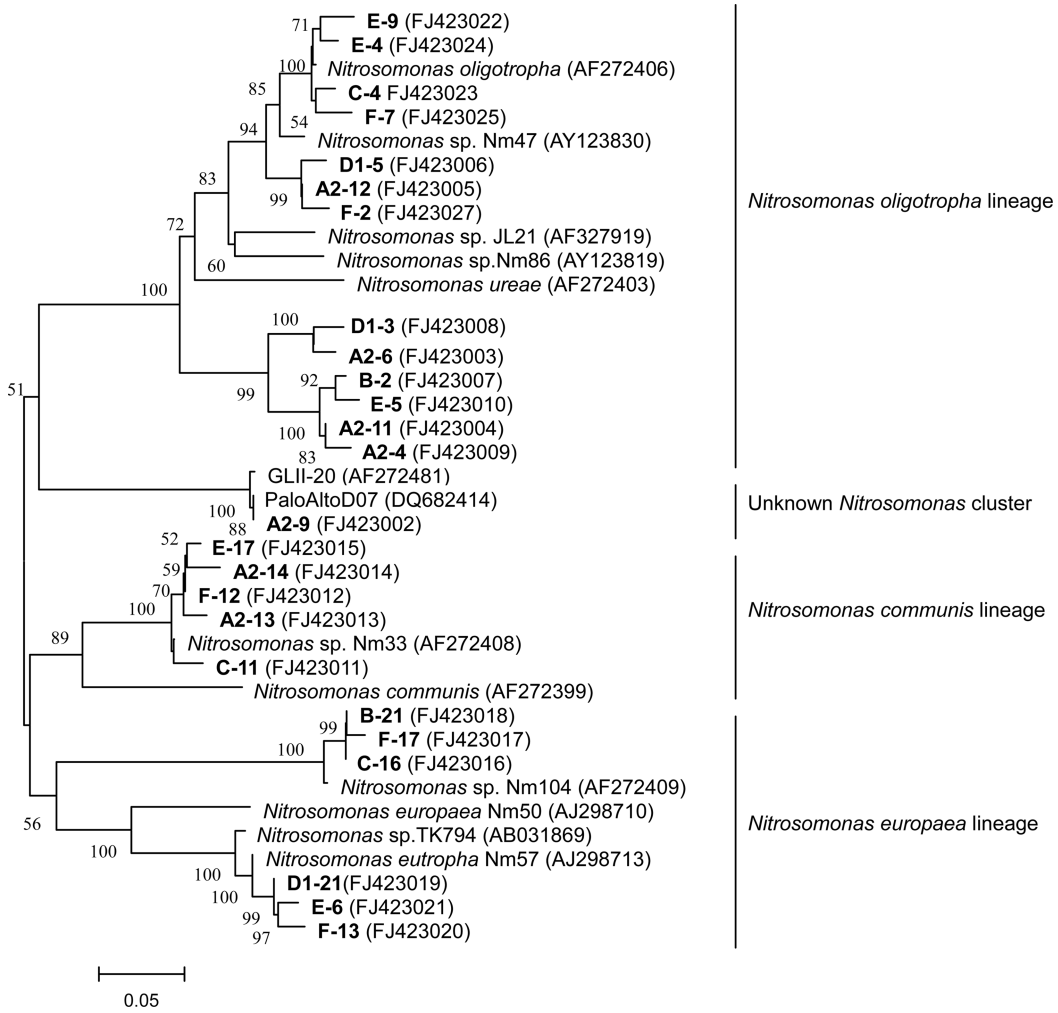

Fig. 3 Phylogenetic tree showing the relationships of partial *amoA* gene sequences to reference sequences from the GenBank database. The tree was constructed with the neighbor-joining method using 450 nucleotide positions. Bootstrap values, shown at nodes where the value is greater than 50, are based on 100 trials. The scale bar indicates 5% sequence difference.

3.3 AOB communities in different scale systems

Pilot-scale reactors are commonly used to simulate full-scale plants because they permit the effects of defined experimental perturbations to be evaluated. Ideally, pilot- and full-scale reactors should possess similar microbial populations. In our study, a relatively high similarity degree of AOB populations in the pilot- and full-scale wastewater reactors was observed, although the full-scale WWTP showed a larger number of abundant AOB species than the pilot-scale reactor. This finding agrees with previous studies (Rowan et al., 2003). Hallin et al. (2005) suggested that the level of AOB diversity relates to the operational stability of the process; there have been indications that plants with a low diversity of a given functional group are more prone to process failure than plants showing a higher diversity of the same bacterial group (Rowan et al., 2003). Therefore, selection for more AOB species may reduce the vulnerability of the plant to process failure, which would be advantageous for process control.

3.4 AOB communities responding to different sources of influent

In our study, activated sludges in WWTPs treating domestic wastewater contained a higher AOB diversity than that in WWTPs receiving mixed domestic and industrial wastewater. Sangchul et al. (2005) also demonstrated that domestic WWTPs exhibited a higher level of bacterial diversity than industrial WWTPs. The reason may that some constituents of the industrial wastewaters are toxic to certain bacteria.

3.5 Phylogenetic analysis

Phylogenetic analysis of cloned *amoA* genes clearly showed that all the sequences were closely related to *Nitrosomonas* spp., not to *Nitrosospira* spp. This result is consistent with most previous studies (Dionisi et al., 2002; Persson et al., 2002; Rowan et al., 2002, 2003; Egli et al., 2003; Park and Noguera, 2004; Hallin et al., 2005; Kelly et al., 2005; Limpiyakorn et al., 2005, 2007; LaPara and Ghosh, 2006), but is in contrast to a few studies which found *Nitrosospira* spp. to be the dominant AOB (Schramm et al., 1998; Rowan et al., 2003).

In pure-culture studies, fast-growing *Nitrosomonas*, such as *N. europaea*, can have a maximum specific growth rate (μ_{max}) as high as 0.088/hr, whereas *Nitrosospira* sp. has a μ_{max} ranging from 0.033 to 0.035/hr (Siripong

and Rittmann, 2007). This growth advantage may favor *Nitrosomonas* over *Nitrosospira* as the prevailing species in activated sludge.

All samples from the eight wastewater treatment systems showed a peak at 354 bp, a signature peak of the *N. oligotropha* cluster. AOB belonging to this cluster are often recovered from freshwater, sediments, soils, WWTPs and other environments (Regan et al., 2002; Hallin et al., 2005; Limpiyakorn et al., 2005; LaPara and Ghosh, 2006; Qin et al., 2008).

A study of isolates of the *N. oligotropha* cluster revealed that they have very low K_s values for free ammonia, ranging from 0.027 to 0.059 mg/L (Lydmark et al., 2007). This does not seem to apply to the *N. oligotropha* strain found here, as the influent ammonium concentrations of the systems were above 36.6 mg/L. Recent findings suggested that the ecological and physiological characteristics of AOB within the *N. oligotropha* cluster are somewhat variable (Lydmark et al., 2007). Factors such as ammonia affinity, oxygen affinity, sensitivity to salt and nitrite may differ between strains. The effect of each environmental factor on members within this cluster needs further study, in order to better understand the implications of the presence of various sequence types (Limpiyakorn et al., 2005).

We observed a peak of 291 bp from systems C, D1, E, and F, which indicates the presence of *N. europaea* cluster. The strains of this cluster have been widely observed in WWTPs (Egli et al., 2003; Park and Noguera, 2004; Hallin et al., 2005; Limpiyakorn et al., 2005; Lydmark et al., 2007; Siripong and Rittmann, 2007). The significant physiological feature of this cluster is a high ammonia K_s value ranging from 0.42 to 0.85 mg/L (Limpiyakorn et al., 2005) suggesting that they prefer eutrophic environments. The ammonia concentrations in the influent of systems with the 291 bp peak (C, D1, D2, E and F) were not significantly different from those of other systems (A1, A2, B and D3) (Table 1). However, the *N. europaea* cluster was present only in some systems (C, D1, D2, E and F). Therefore, ammonia concentration was not responsible for the appearance of *N. europaea* cluster in these systems. There may be a variation in ecophysiology within *N. europaea* cluster, just as in *N. oligotropha* cluster. The factors that caused these systems to select for *N. europaea* cluster need further study.

We also found TF peaks at 491 bp in all samples, which corresponds to *N. communis cluster*. *N. communis* has previously been detected in soils, activated sludge and biofilm systems (Purkhold et al., 2000; Gieseke et al., 2001, 2005). Their relatively high ammonia K_s value of 0.20–0.60 mg/L (Koops and Pommerening-Röser, 2001) suggest that they are better adapted to high ammonia concentrations than, for example, *N. oligotropha*. This physiological property may favor the *N. communis* cluster's presence in the systems, as the ammonia concentrations in the influent of all systems are higher than 41.0 mg/L. Recently, Limpiyakorn et al. (2005) also suggested that *N. communis* may have an advantage in environments with fluctuating oxygen levels or in the absence of oxygen. However, Park et al. (2008) demonstrated that DO concentration did not influence the AOB community, but rather the activity of AOB. Furthermore, Lydmark et al. (2007) concluded that there are not yet enough data to allow correlations between AOB clusters and oxygen levels.

We also observed some less abundant T-RFs of 85, 205, 334, 439, 483 bp from some systems. These T-RFs have not been identified from the clones of *amoA* genes. These infrequent T-RFs are also not expected fragment sizes derived from TAP T-RFLP in silico analysis in Ribosomal Database Project version 8.1 (http://rdp.cme.msu.edu). They could belong to uncharacterized AOB. We speculate that the number of AOB species is greater than those represented in the database.

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