Distribution of ammonia-oxidizing bacteria in sewage activated sludge: analysis based on 16S rDNA sequence

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Abstract This study carried out analysis of ammonia-oxidizing bacteria (AOB) communities in 12 sewage activated sludge systems standing in eight sewage treatment plants located in Tokyo. The systems were different in the treatment process configuration: anaerobic/anoxic/aerobic (A2O), anaerobic/aerobic (AO), and conventional activated sludge (AS) processes. AOB communities were analyzed by sequences of 16S rDNA amplicons, which were separated by denaturing gradient gel eletrophoresis (DGGE) after specific polymerase chain reaction (PCR) amplification. The results demonstrated that low ammonium concentrations in the influents of the 12 sewage activated sludge systems resulted in the dominance of *Nitrosomonas oligotropha*-like sequences. Further, *Nitrosomonas europaea*- and *Nitrosomonas cryotolerans*-like sequences were recovered from only one A2O system of which the influent contained higher ammonium and chloride concentrations than those of other systems. *Nitrosomonas communis*-like sequences were found in every A2O and AO system, but mostly not found in every AS system. In summary, influent characteristics and treatment process configuration affected the AOB communities in the 12 sewage activated sludge systems.

Keywords Activated sludge; ammonia-oxidizing bacteria; microbial community; sewage treatment

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Introduction

Nitrification, the key process of biological nitrogen removal in wastewater treatment systems, is the two-step process concerning two phylogenetically unrelated groups of obligate chemolithotrophic bacteria: ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). AOB first oxidize ammonia to nitrite, and nitrite subsequently is oxidized to nitrate by NOB. Due to the slow growth rate of AOB, and their high sensitivity to several environmental factors, ammonia oxidation is generally the rate-limiting step of biological nitrogen removal in wastewater treatment systems (Wagner *et al*., 1995). Therefore, a better understanding of the microbiology and ecology of AOB in wastewater treatment systems is necessary for the enhancement of treatment performance and control (Okabe *et al*., 1999).

Recently, molecular tools have been developed to incorporate 16S rRNA and *amoA* genes of AOB. As they allow observation of AOB without concerning traditional cultivation dependent techniques, the molecular techniques have been introduced to study AOB in many environments. Although AOB communities were previously observed in many wastewater treatment systems (Mobarry *et al*., 1996; Ballinger *et al*., 1998; Juretschko *et al*., 1998; Wagner *et al*., 1998), those in sewage activated sludge systems are not clearly understood.

Therefore, this study carried out analysis of ammonia-oxidizing bacteria (AOB) communities in sewage activated sludge systems. Because it allows clarification of AOB communities at the species level, specific polymerase chain reaction (PCR) amplification, denaturing gradient gel electrophoresis (DGGE), cloning, and sequencing of 16S rRNA

gene were used to reveal the AOB communities. Effects of influent characteristics and treatment process configuration of the 12 sewage activated sludge systems on the AOB communities were mainly focused on.

Methods

Sewage activated sludge samples

Samples were collected from 12 sewage activated sludge systems standing in eight sewage treatment plants located in Tokyo. The systems were different in treatment process configuration: anaerobic/anoxic/aerobic (A2O), anaerobic/aerobic (AO), and conventional activated sludge (AS) processes. Table 1 shows treatment process configuration, influent characteristics, and removal efficiencies of the 12 sewage activated sludge systems. After sampling, activated sludge of approximately 2 mg MLSS was transferred to 1.7 ml eppendorf tubes and centrifuged at 14,000 g for 10 min. The supernatant was removed, and the pellet was kept at –20°C.

DNA extraction

FastDNA SPIN kits for soil (Bio 101, Vista, CA, USA) were used for DNA extraction of activated sludge samples with a small modification at the initial step: 1 ml of sodium phosphate buffer solution was added to and mixed with the sample, and then the tube was sonicated for 30 s on ice. The other steps followed the manufacturer's instructions.

PCR amplification, DGGE, cloning, and sequencing

PCR amplification of 16S rRNA gene was carried out by the primer set, CTO189f and CTO654r (Kowalchuk *et al.*, 1997), specific for β-subdivision AOB. The extracted DNA of activated sludge samples was PCR-amplified by the primer set with an additional GC-clamp. DNA eluted from bands excised from DGGE gels and colonies directly picked after cloning were PCR-amplified by the primer set without an additional GC-clamp. The PCR mixture was prepared with Ampli*Taq* Gold DNA polymerase (PE Applied Biosystems, CA, USA) following the manufacturer's instructions with 1 pmol of each primer. The PCR condition was carried out as described previously (Nicolaisen and Ramsing, 2002) in a T3 thermocycler (Biometric, Gottingen, Germany).

DGGE was performed according to the modification of Kurisu *et al.* (2002). The denaturant gradient of urea and formamide was 35–55%. The gel was run for 16 h at 60°C and 75 V. Strong bands were excised and immersed in 30 µl sterilized water. DNA was recovered from the gel by freeze–thawing three times.

The target DNA fragments recovered from DGGE gel were purified by cloning using pT7Blue T-Vector (Novagen, Darmstadt, Germany) and DH5-α competent cells (Toyobo, Tokyo, Japan) following the manufacturer's instructions.

Small DNA fragments and excess primers were removed from the template DNA by Microcon spin columns (Millipore, Tokyo, Japan). The sequencing reaction was performed

Table 1 Treatment process configuration, influent characteristics, and removal efficiencies of the 12 sewage activated sludge systems

Parameter	Unit	System											
		A	B1	B2	B3	C	D	E	F1	F2	G1	G2	H
System configuration		A2O	A2O	AO	AS	AO	AO	AO	AS	AS	AS	AS	AS
BOD-removal	%	99	99	99	99	96	100	98	98	98	99	95	97
NH_4^+-N-removal	%	100	98	97	97	41	100	85	98	100	100	40	7
NH_4^+-N in the influent	mg/l	26.4	15.7	15.7	15.7	20.4	17.5	13.7	15.2	16.5	17.8	11.5	15.8
Cl^- in the influent	mg/l	180	80	80	80	65	62	90	–	–	94	98	72

with 20 ng of the template DNA, ABI Big Dye Terminator kit version 3.1 (PE Applied Biosystems), and the primer set described above. After removal of excess primers and dye terminators by Centri-sep spin columns (Applied Biosystem, CA, USA), the products were analyzed in an ABI 310 DNA sequencer (Applied Biosystem, CA, USA).

Results and discussion

Figure 1 shows a DGGE image of PCR-amplified products of samples taken from the 12 sewage activated sludge systems. The denaturant gel concentration ranging from 40 to 50% was illustrated in the DGGE image because all DGGE bands closely related to AOB were found in this concentration range. A phylogenetic tree was constructed with the sequences of DGGE bands analyzed in this study and the sequences available in the database of β-subdivision AOB and related non-AOB, which were used as outgroup sequences (Figure 2). Related AOB-like sequences found in the 12 sewage activated sludge systems were summarized in Table 2.

As all sequences analyzed in this study were closely related to *Nitrosomonas* spp, *Nitrosomonas* spp., rather than *Nitrosospira* spp., were the major AOB in the 12 sewage activated sludge systems. Because *Nitrosomonas* spp. often are observed in wastewater treatment systems, a few previous studies were in agreement (Mobarry *et al*., 1996; Ballinger *et al*., 1998; Jurestschko *et al*., 1998; Wagner *et al*., 1998; Okabe *et al*., 1999; Gieseke *et al*., 2001).

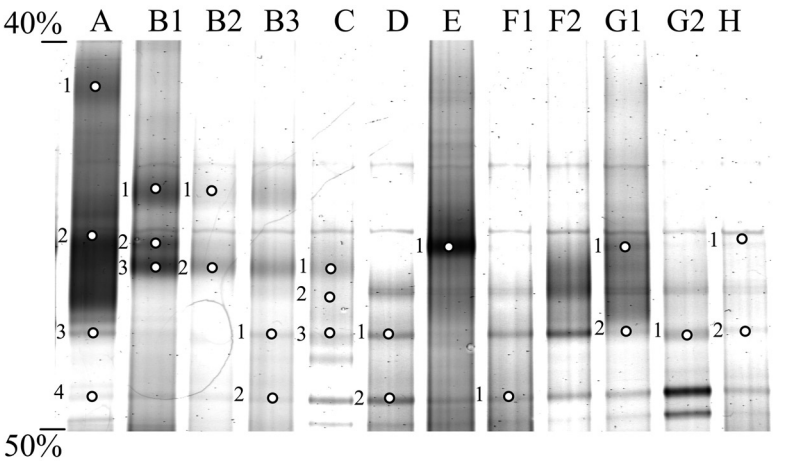

Figure 1 DGGE image of PCR-amplified products of samples taken from the 12 sewage activated sludge systems. System names were depicted above their lanes, and DGGE bands analyzed were marked and numbered

Table 2 Summary of related AOB-like sequences found in the 12 sewage activated sludge systems

Sequence		System											
		A	B1	B2	B3	C	D	E	F1	F2	G1	G2	H
Nitrosomonas communis-like		+	+	+	+	+	+	+					
Nitrosomonas europaea-like		+											
Nitrosomonas cryotolerans-like		+											
Nitrosomonas oligotropha-like	O–1										+		+
	O–2		+	+	+								
	O–3				+	+			+	+		+	+
	O–4	+			+	+	+						
	O–5		+	+	+	+	+	+	+	+	+	+	

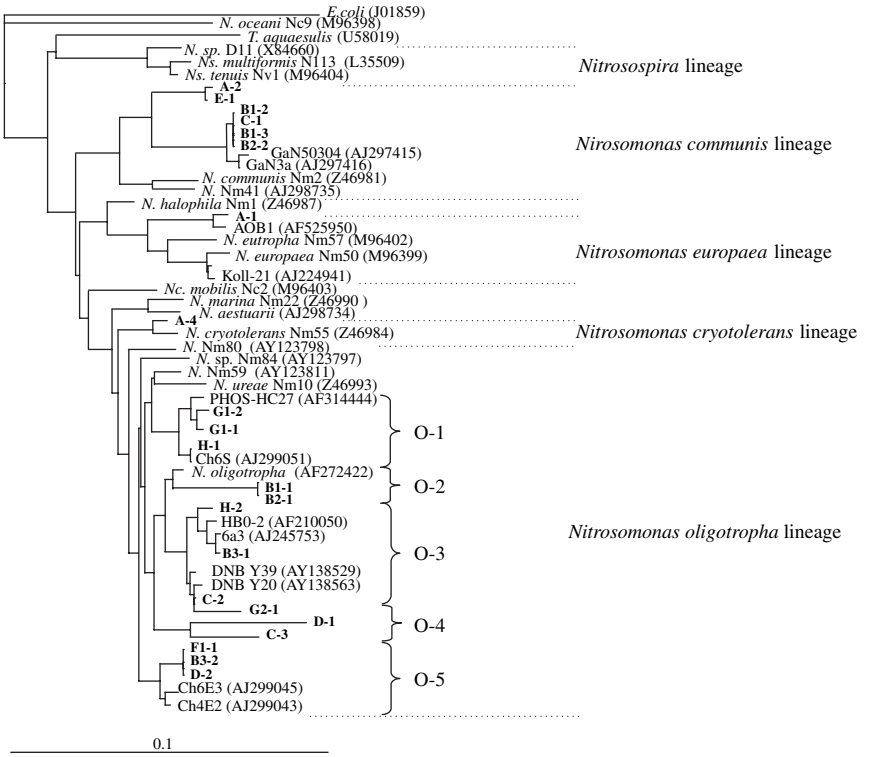

Figure 2 Phylogenetic tree based upon partial 16S rRNA gene sequences of β-subdivision AOB. AOB genus abbreviations are *N.* for *Nitrosomonas*, *Nc.* for *Nitrosococcus*, and *Ns.* for *Nitrosospira*. DGGE bands analyzed in this study are shown in bold

Among the *Nitrosomonas* spp., *Nitrosomonas oligotropha*-like sequences were recovered from every sewage activated sludge system. *Nitrosomonas oligotropha*-like sequences often are found in low-ammonia environments, for example freshwater sediment (Bollmann and Laanbroek, 2001), chloraminated drinking water distribution systems (Regan *et al.*, 2002; Regan *et al.*, 2003), and wastewater treatment systems treating low-ammonia-concentration wastewater (Ballinger *et al.*, 1998; Gieseke *et al.*, 2001). Therefore, low ammonium concentrations in the influents (11–27 mg/l NH_4^+-N) of the 12 sewage activated sludge systems may result in the dominance of *Nitrosomonas oligotropha*-like sequences.

From other systems, system A contained a distinct AOB community. *Nitrosomonas europaea*- and *Nitrosomonas cryotolerans*-like bacteria comprised the AOB community in system A only. By molecular-level investigations, *Nitrosomonas europaea*-like bacteria previously were found in wastewater treatment systems treating rich ammonia wastewater (Logemann *et al.*, 1998; Pynaert *et al.*, 2003) and alkaline, high-salinity environments (Ward *et al.*, 2000). For these reasons, the existence of *Nitrosomonas europaea*-like bacteria in the environment related to ammonium or salt concentrations. As the influent of system A contained higher ammonium and chloride concentrations than other systems, this distinct characteristic of the influent may result in the presence of the *Nitrosomonas europaea*- and *Nitrosomonas cryotolerans*-like bacteria in only system A.

As they were present in every AO and A2O system, but mostly absent from every AS system, *Nitrosomonas communis*-like bacteria were found almost exclusively in AO and A2O systems. A previous study (Gieseke *et al.*, 2001) also reported on the *Nitrosomonas communis*-like bacteria in the phosphate-removing biofilm of a sequencing batch reactor.

Therefore, the presence of the *Nitrosomonas communis*-like bacteria may relate to the treatment process configuration, especially where the oxygen level fluctuated.

Conclusions

AOB communities in 12 sewage activated sludge systems were analyzed. It was found that characteristics of the influent and treatment process configuration of the sewage activated sludge systems affected the AOB communities.

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