

Communities of Ammonia-oxidizing Archaea and Bacteria in Enriched Nitrifying Activated Sludge

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Abstract—In this study, communities of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) in nitrifying activated sludge (NAS) prepared by enriching sludge from a municipal wastewater treatment plant in three continuous-flow reactors receiving an inorganic medium containing different ammonium concentrations of 2, 10, and 30 mM $\text{NH}_4^+\text{-N}$ (NAS2, NAS10, and NAS30, respectively) were investigated using molecular analysis. Results suggested that almost all AOA clones from NAS2, NAS10, and NAS30 fell into the same AOA cluster and AOA communities in NAS2 and NAS10 were more diverse than those of NAS30. In contrast to AOA, AOB communities obviously shifted from the seed sludge to enriched NASs and in each enriched NAS, communities of AOB varied particularly. The seed sludge contained members of *N. communis* cluster and *N. oligotropha* cluster. After it was enriched under various ammonium loads, members of *N. communis* cluster disappeared from all enriched NASs. AOB with high affinity to ammonia presented in NAS 2, AOB with low affinity to ammonia presented in NAS 30, and both types of AOB survived in NAS 10. These demonstrated that ammonium load significantly influenced AOB communities, but not AOA communities in enriched NASs.

Keywords—ammonia-oxidizing bacteria, ammonia-oxidizing archaea, nitrifying activated sludge.

I. INTRODUCTION

AMMONIA oxidation is considered to be the rate-limiting step of the removal of nitrogen in global nitrogen cycle as well as wastewater treatment plants. It was believed for long time that this process was mediated by only a group of chemolithoautotrophic ammonia-oxidizing bacteria (AOB). Until the year 2004, it was first time revealed that autotrophic ammonia oxidation is not only restricted to the domain *Bacteria*, but also domain *Archaea*. The molecular evidences demonstrated the presence of an ammonia monooxygenase (*amoA*)-like gene on an archaeal scaffold and indicated the potential role of archaea in nitrification process in the ocean [1]. Then, Treusch [2] discovered gene that potentially encode ammonia monooxygenase (AMO), a key enzyme in ammonia oxidation. The ultimate confirmation of ammonia-oxidizing archaea (AOA) activity was achieved by the first cultivation

and isolation of autotrophic ammonia-oxidizing marine archaeon named *Nitrosopumilus maritimus* [3]. There are some evidences, showing that AOA are more abundant than AOB in marine [4] and soil environments [5]. Beside marine and soil environments, AOA have been detected in activated sludge bioreactors by using specific PCR primers targeting archaeal *amoA* gene [6].

While lacking of scientific information on AOA, many studies showed the distribution patterns of distinct AOB species in the environments reflecting the physiological properties of AOB isolates observed in the laboratory [7]. Among several factors, ammonia, as the essential energy source, is the most important factors affecting AOB communities in the environments. However, so far, physiological properties information on AOA is not yet discovered. Only rough information could be obtained from few molecular evidences from natural environments.

In this study, we enriched nitrifying activated sludge (NAS) feeding with inorganic medium containing different ammonium concentrations (2, 10 and 30 mM $\text{NH}_4^+\text{-N}$). AOA communities were investigated by specific Polymerase Chain Reaction (PCR) amplification followed by constructing clone library and sequencing of archaeal *amoA* gene, while AOB communities were analyzed by specific PCR amplification followed by Denaturing Gel Gradient Electrophoresis (DGGE) and sequencing of bacterial 16S rRNA gene.

II. MATERIALS AND METHODS

A. Enrichment of Nitrifying Activated Sludge

Sludge taken from a municipal WWTP was enriched in three continuous-flow reactors receiving inorganic medium containing different ammonium concentrations of 2, 10 and 30 mM $\text{NH}_4^+\text{-N}$. (NAS2, NAS10, and NAS30, respectively). Inorganic medium was introduced into all NASs at a fixed dilution rate of 0.01 hr^{-1} [8].

B. Analysis of Ammonia-oxidizing Archaeal Communities

Primers Arch-*amoA*F and Arch-*amoA*R [9] were used to amplify 635-bp of archaeal *amoA* gene fragments. The PCR mixture was prepared using Takara polymerase (Takara Bio Inc, Japan). Triplicate PCR products were pooled and purified by gel electrophoresis using a NucleoSpin Extract II Kit (Clontech Laboratories, Inc., USA). Then, the purified PCR products were cloned using the pGEM-T Easy vector system (Promega, Madison, WI). For each sample, 10-30 clones were

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randomly selected for sequencing.

C. Analysis of Ammonia-oxidizing Bacterial Communities

Primers CTO189f and CTO654r [11] were used to amplify 465-bp of 16S rRNA gene fragment of *Betaproteobacteria*. We use 8% polyacrylamide gels, and the urea-formamide denaturant gradient was 30–70%. Gels were run on the D Code system (Bio-Rad Laboratories, Hercules, CA, USA) for 16 h at 60 °C and 75 V. Prominent bands were excised and dissolved in 30µl sterilized water. DNA was recovered from the gel by freeze–thawing three times.

III. RESULTS AND DISCUSSION

A. Enrichment of Nitrifying Activated Sludge

Three enriched NAS feeding with inorganic medium containing different ammonium concentrations (2, 10, and 30 mM NH₄⁺-N) were operated for 60 days. During operation, ammonium, nitrite, and nitrate concentrations were monitored. Ammonium concentrations in all three reactors reached the steady-state conditions after certain periods of operation. In all cases, ammonium was completely oxidized. After 60 days of operation, sludge samples were collected to analyze for communities of AOA and AOB.

B. Communities of Ammonia-oxidizing Archaea

In total 88 clones, analyzed for sequencing, were tested for sequence similarity using blast program (Table 1). Results suggested that all analyzed showed 88 – 98% identity at nucleotide level to previously reported AOA *amoA* gene sequences. All 88 AOA *amoA* sequences were categorized into 30 OTUs. The amounts of the sequences of each OTU were displayed by the number in parentheses of Table 1.

For seed sludge sample, ten clones randomly selected fell into 2 clusters: 7 clones in cluster K and 3 clones in cluster I. For NAS 2, 21 clones were randomly selected for sequencing. All 21 cloned analyzed could be divided into 3 AOA clusters: 19 clones in cluster K, 1 clone in cluster E, and 1 clone in cluster F. In the case of NAS 10, twenty-six randomly selected clones were divided into 4 AOA clusters which were 23 clones for cluster K, 1 clone for cluster B, 1 clone for cluster E, and 1 clone for cluster G. All randomly selected clones of NAS 30 were members of cluster K only.

TABLE I CLOSELY RELATED SEQUENCES OF AOA *amoA* GENE FRAGMENTS

Sample	Clone	Score	Percent Identity	Gap	Accession No. of closely related sequence	closely related sequence
Seed Sludge	AOA-S-1 (5)	957	541/552 (98%)	2/552 (0%)	EU239976	Clone MamSp.H08
	AOA-S-2 (1)	946	539/552 (97%)	2/552 (0%)	EU239976	Clone MamSp.H08
	AOA-S-3 (1)	953	548/563 (97%)	4/563 (0%)	EU239976	Clone MamSp.H08
	AOA-S-4 (1)	833	527/564 (93%)	3/564 (0%)	FJ227760	Clone WBM050405_45P2A1
	AOA-S-5 (1)	693	473/537 (88%)	2/537 (0%)	FJ227760	Clone WBM050405_45P2A1
	AOA-S-6 (1)	819	518/555 (93%)	1/555 (0%)	FJ227760	Clone WBM050405_45P2A1
NAS 2	AOA-NAS2-1 (5)	976	552/563 (98%)	3/563 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS2-2 (9)	981	553/563 (98%)	3/563 (0%)	EU239976	Clone MamSp.H08

NAS 10	AOA-NAS2-3 (2)	977	553/564 (98%)	3/564 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS2-4 (1)	955	549/564 (97%)	4/564 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS2-5 (1)	941	546/563 (96%)	5/563 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS2-6 (1)	815	537/583 (92%)	7/583 (1%)	FJ227153	Clone 3_15
	AOA-NAS2-7 (1)	992	557/566 (98%)	3/566 (0%)	EU022958	Clone HB C 0604 C02
	AOA-NAS2-8 (1)	673	497/561 (88%)	1/561 (1%)	EU239976	Clone MamSp.H08
	AOA-NAS10-1 (14)	979	553/564 (98%)	2/564 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS10-2 (6)	983	554/564 (98%)	3/564 (0%)	EU239976	Clone MamSp.H08
NAS 30	AOA-NAS10-3 (1)	955	549/564 (97%)	3/564 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS10-4 (1)	966	551/564 (97%)	3/564 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS10-5 (1)	966	557/573 (97%)	3/573 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS10-6 (1)	963	556/573 (97%)	2/573 (0%)	EU590230	Clone BGA-781
	AOA-NAS10-7 (1)	896	539/564 (95%)	7/564 (1%)	DQ501174	Clone MX_2_OCT_29
	AOA-NAS10-8 (1)	965	566/587 (96%)	4/587 (0%)	EU025177	Clone S18-A-16
	AOA-NAS30-1 (19)	990	559/570 (98%)	1/570 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS30-2 (5)	992	558/569 (98%)	1/569 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS30-3 (1)	970	552/565 (97%)	2/565 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS30-4 (1)	972	553/565 (97%)	5/565 (0%)	EU239976	Clone MamSp.H08
NAS 30	AOA-NAS30-5 (1)	963	551/565 (97%)	3/565 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS30-6 (1)	970	551/563 (97%)	3/563 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS30-7 (1)	987	560/572 (97%)	3/572 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS30-8 (1)	961	551/566 (97%)	2/566 (0%)	EU239976	Clone MamSp.H08
	AOA-NAS30-9 (1)	961	551/566 (97%)	2/566 (0%)	EU239976	Clone MamSp.H08

Number in parenthesis indicated amounts of AOA *amoA* sequences showing 100% identity

TABLE II SUMMARY OF AOA FOUND IN SEED SLUDGE AND ENRICHED NASS

Cluster	Seed sludge	NAS 2	NAS 10	NAS 30
B			✓	
E		✓	✓	
F		✓		
G			✓	
I	✓			
K	✓	✓	✓	✓

✓, present

It seemed that AOA communities in NAS 2 and NAS 10 were more diverse than NAS 30. AOA communities were more stable by being less influence by ammonium loads than the AOB communities. Most of the clones from seed sludge (7 out of 10), NAS 2 (19 out of 21), NAS 10 (23 out of 26), and NAS 30 (30 out of 30) fell only in the same AOA cluster K. In the case of seed sludge as mentioned above, seven out of ten clones related closely to those obtained from Mammoth hot spring sediment (cluster K) [13] while another 3 clones related to AOA found in sediment (cluster I) (unpublished). Interestingly, no clone from the seed sludge related closely to those in the only study of AOA in WWTPs [6] in spite the seed sludge in our study was taken from a municipal WWTPs (activated sludge). Other than cluster K, clones from NAS 2 related to the clones retrieved from coastal marine sediment

(cluster E) (unpublished) and coastal sediment (cluster F) [14]. In addition, three clones from NAS 10 were closely related to those obtained from estuarine sediment (cluster E), sediment (cluster B), and soil (cluster G) (unpublished).

C. Analysis of Ammonia-oxidizing Bacterial Communities

In total 8 bands, analyzed for sequencing, were tested for sequence similarity using blast program (Table 3). Results suggested that all analyzed sequences showed 96 -99% identity at nucleotide level to the previous reported sequences in the database. AOB found in each sample were summarized in Table 4.

Results revealed the shift in AOB communities from a seed sludge to enriched NAS, and the communities of AOB in each NAS varied significantly. AOB in a seed sludge related closely to *N. communis* and *N. oligotropha* cluster respectively. Although members of *N. communis* cluster were reported for their moderate affinity to free ammonia ($K_s = 14$ to $43 \mu\text{M}$;

representatives in natural freshwater environments. Moreover, *N. oligotropha* are the most common AOB found in WWTPs with low ammonium loads [15]. It is not surprised to find these two AOB cluster in the seed sludge, as the seed sludge was taken from a municipal WWTP receiving low ammonium load. Band analyzed for NAS 2 closely related to unknown *Nitrosomonas* cluster. Bands analyzed from NAS 10 were found to be related to unknown *Nitrosomonas* cluster. While, another band of this sample was closely related to *N. europaea* cluster. Surprisingly, groups of AOB exhibiting low and high affinity to ammonia was found in this NAS that received the moderate ammonium load. Band from NAS 30 was affiliated to *N. europaea* cluster. These AOB are low in affinity to ammonia ($K_s > 30 \mu\text{M}$; [12]). They are often found in wastewater with high ammonium loads, eutrophic freshwaters, or fertilized soil [12].

AOB can be divided into two groups which are AOB with high and low affinity to free ammonia. AOB with high affinity to ammonia could be retrieved from NAS 2 and NAS 10. Whereas AOB with low affinity to ammonia could be recovered from NAS 10 and NAS 30. AOB communities in NAS 10 were the mixture of AOB of high and low affinity to ammonia. This may be because of the moderate ammonium load supplied to the NAS 10. This demonstrated that ammonium load is the important factor, selecting the communities of AOB in the enriched NAS and the selection is based on the physiological properties (ammonia affinity) reported for the isolated AOB cultures in the previous studies.

IV. CONCLUSION

This study revealed that ammonium load was confirmed to be the major factor selecting communities of AOB. AOB with high affinity to ammonia presented in NAS 2, AOB with low affinity to ammonia presented in NAS 30, and both strains can survive in NAS 10. In contrast to AOB, AOA communities were more stable under ammonium load variation. Almost all AOA *amoA* sequences from all enriched NASs fell in the same cluster (cluster K).

TABLE III CLOSELY RELATED SEQUENCES OF AOB 16S rRNA GENE FRAGMENTS

Sample	Band	Score	Percent Identity	Gap	Accession No. of closely related sequence	closely related sequence
Seed sludge	AOB-S-1	787	436/443 (98%)	1/443 (0%)	AB222811	DGGE 0NO2c-3
	AOB-S-2	806	441/444 (99%)	0/444 (0%)	AJ297415	Clone GaN50304
	AOB-S-3	811	441/442 (99%)	0/442 (0%)	AJ297415	Clone GaN50304
NAS 2	AOB - NAS2-1	712	403/411 (98%)	3/411 (0%)	AM29553 2	Clone Nm 271104_1
NAS 10	AOB - NAS10-1	813	452/457 (98%)	3/457 (0%)	AY123795	<i>Nitrosomonas europaea</i>
	AOB - NAS10-2	739	431/446 (96%)	6/446 (1%)	AM29553 2	Clone Nm 271104_1
	AOB - NAS10-3	728	405/410 (98%)	1/410 (0%)	AM29553 2	Clone Nm 271104_1
NAS 30	AOB - NAS30-1	830	455/458 (99%)	1/458 (0%)	AL954747	<i>Nitrosomonas europaea</i> ATCC 19718

TABLE IV SUMMARY OF AOB FOUND IN SEED SLUDGE AND ENRICHED NASs

Cluster	Seed sludge	NAS 2	NAS 10	NAS 30
<i>Nitrosospora</i> cluster				
unknown <i>Nitrosomonas</i> cluster		✓	✓	
<i>N. cryototerans</i> cluster				
<i>N. europaea</i> - <i>Nc. mobilis</i> cluster			✓	✓
<i>N. communis</i> cluster	✓			
<i>N. marina</i> cluster				
<i>N. oligotropha</i> cluster	✓			

✓, present

Koops *et al.*, 2003), they were often recovered from wastewater treatment systems receiving low ammonium loads [15]. *N. oligotropha* cluster are well known to be common AOB in municipal WWTPs [15] as they exhibited high affinity to ammonia ($K_s = 1.9$ to $4.2 \mu\text{M}$; [12]), meaning that they prefer low ammonia habitats. Member of *N. oligotropha* cluster are absolute majority strains originating from oligotrophic freshwaters and generally the dominant AOB

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